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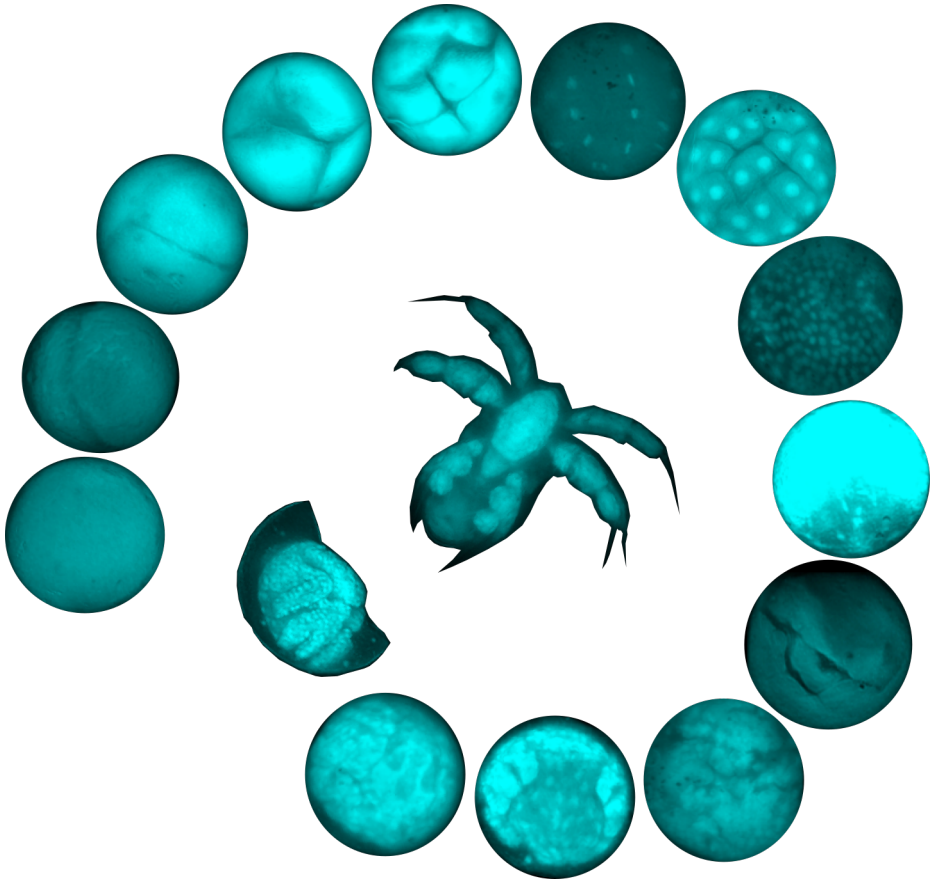

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This thesis has been submitted to the *Doctoral School of Science and Environment*,
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Preface

This Ph.D. dissertation is a part of the project: *Acartia tonsa* Molecular PHysiology Implementation of novel and fast tools to assess COPepod physiological states (AMPHICOP). The project was supported by a research grant (No. 8689) from VILLUM FONDEN.

Roskilde University Department of Science and Environment and Graduate School of Environmental Stress Studies (GESS) was responsible for hosting me during the Ph.D. journey.

University of Connecticut Department of Marine Sciences and Professor Ann Bucklin hosted me during my research stay in the U.S.

The research group included following members:
Ph.D. student Birgitte Nilsson, Roskilde University
Professor Benni Winding Hansen, Roskilde University
Post-doctoral fellow Tue Sparholdt Jørgensen, Roskilde University
Professor Lars Hestbjerg Hansen, Aarhus University
Professor Ann Bucklin provided supervision at the University of Connecticut.

Research field: Molecular stress physiology

Abstract

The human population is growing which causes major changes in marine ecosystems in terms of thermal stress, acidification, and pollution. This is and will inevitably have a negative impact on marine plants and animals.
Copepods are intermediaries of the marine food web, providing a link of energy transfer from phytoplankton to higher trophic levels. If copepods suffer due to environmental changes, it will affect the whole food web negatively. It is, thus, important to understand how stressors are affecting copepods.

The aim of the present Ph.D. dissertation is to provide new information for understanding how environmental stress affects the calanoid copepod, Acartia tonsa, at the transcriptional level. Targeted - (i.e. real-time quantitative PCR) and transcriptome-wide methods for examining gene expression are able to give an early indication of when organisms experience a condition as stressful.
The three produced manuscripts of the present Ph.d. dissertation covers different aspects of molecular stress physiology in *Acartia tonsa*:

Producing *Acartia tonsa* in high densities, as live feed for fish-larvae, is a major obstacle in the aquaculture industries. In manuscript I, *Acartia tonsa* was exposed to very high densities (up to 10,000 ind. L^{-1}) for 12 h. The short-term exposure to elevated densities did not cause any significant changes in swimming behavior, respiration, or gene expression of *heat shock protein 70*, and *90 kDa*, as well as *ferritin*.

In gene expression studies, handling has the potential of affecting the transcription of stress-related biomarkers. In manuscript II, the whole-transcriptome response towards handling stress and salinity shock (i.e., positive control) were examined in comparison to a negative control for *Acartia tonsa*. The effect of the treatments were evaluated 15 min and 24 h post-exposure, since it was not clear when to expect a transcriptional response. Handling did affect the transcriptional pattern in *Acartia tonsa*, especially after 24 h. Among the differential expressed genes, a number of commonly-used copepod biomarkers were identified. Thus, the biomarkers for stress-related studies should be chosen carefully.

Acartia tonsa is capable of producing embryonic quiescence in response to unfavorable environmental conditions. Manuscript III, provides a detailed description of subitaneous development and quiescence in *Acartia tonsa* eggs. Furthermore, it was demonstrated that timing of quiescence determines egg viability.

Resumé (Danish abstract)

Væksten af den menneskelige population er årsag til store ændringer i det marine økosystem, såsom termisk stress, forsuring og forurening. Dette vil unægtelig resultere i en negativ påvirkning af marine planter og dyr.
Vandlopper er et mellemled i den marine fødekæde, hvor de kobler energi fra fytoplankton til højere trofiske niveauer. Hvis vandlopperne bliver belastet af miljømæssige ændringer vil det påvirke den marine fødekæde negativt.

Formålet med Ph.D. afhandlingen var at bidrage med ny viden til at kunne forstå hvordan miljømæssig stress påvirker vandloppen, *Acartia tonsa*, på gen-niveau. Målrettede – (dvs. real-time quantitative PCR), såvel som "whole-transcriptome" metoder blev benyttet for at undersøge gen-ekspression. Gen-ekspression kan give en tidlig indikation på hvornår en organisme oplever stress.

De tre producerede manuskripter af denne Ph.D. afhandling dækker forskellige aspekter indenfor molekylær stress-fysiologi af *Acartia tonsa*.

Produktionen af *Acartia tonsa* i høje densiteter, til brug som levende foder for fiskelarver, er en stor hindring i akvakultur-industrien. I manuskript I blev *Acartia tonsa* udsat for høje densiteter (op til 10,000 ind. L^{-1}) i 12 timer. Den kortvarige eksponering til øgede densiteter gav ikke nogen signifikante ændringer i svømme-adfærd, respiration, eller gen-ekspression af *heat-shock protein 70*-, og 90 *kDa*, så vel som *ferritin*.

Håndtering af dyr har potentialet til at påvirke gen-ekspression af stress-relaterede biomarkører. I manuskript II blev stress-responsen mod håndterings - og salt stress undersøgt med RNA-sekvensering. Effekten af behandlingerne blev evalueret 15 min og 24 timer efter eksponering da det var uklart hvornår der kunne forventes en respons på det molekylære niveau. Håndtering havde en klar påvirkning på gen-ekspression for *Acartia tonsa*, specielt 24 timer efter eksponering. Blandt de differentierede udtrykte gener blev der identificeret et antal biomarkører som typisk bliver brugt i vandloppe-studier. Valget af biomarkører i gen-ekspressions studier bør derfor vælges nøje for at undgå en håndterings-effekt.

Acartia tonsa er i stand til at inducere et embryonisk hvilestadie, kaldt quiescens, i respons til ugunstige forhold. Manuskript III giver en detaljeret beskrivelse af den subitane udvikling, såvel som quiescens i *Acartia tonsa* æg. Det var desuden demonstreret at timing for induktion af quiescens bestemmer æggenes levedygtighed.

Acknowledgements

My dissertation was about stressing copepods in different ways to uncover what happens at the molecular level – and thereby provide cues to how this tiny creature "works" and copes with its surroundings. But to be honest, sometimes the copepods were stressing me more than I was stressing them.

This work is a part of the AMPHICOP project and was carried out at Roskilde University (Roskilde, Denmark) for three years, with a six months research stay at the University of Connecticut (UConn, Groton, USA).

Many people have supported my journey, and I would like to thank them with gratitude. Especially I would like to thank:

- Professor Benni W. Hansen for supervision and guidance during this amazing journey.
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- Thanks to my friends and family for being supportive and loving. Especially thanks to my boyfriend, Stefan, and my cousin, Maiken, for always being there for me with open ears and arms.

Table of Contents

List of abbreviations

18S	18S ribosomal RNA
ACT	ß-actin
A. tonsa	*Acartia tonsa*
ATPS	ATP synthase
bp	base pairs
cDNA	complementary DNA
Contig	Contiguous sequence
EFA	Elongation factor α
ER	Endoplasmic Reticulum
gDNA	genomic DNA
DEGs	Differentially Expressed Genes
GO	Gene Ontology
HIST	Histone H3
HSP70	Heat-shock protein 70kDa
HSP90	Heat-shock protein 90kDa
KEGG	Kyoto Encyclopedia of Genes and Genomes
mRNA	messenger RNA
miRNA	microRNA
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
nt	nucleotides
ORF	Open Reading Frame
Real-time qPCR	Real-time quantitative PCR
RNA-Seq	RNA sequencing
rRNA	ribosomal RNA
RUC	Roskilde University
S20	Ribosomal protein S20
SD	Standard deviation
SNP	Single-Nucleotide Polymorphism
tRNA	transfer RNA
UBI	Ubiquitin
UConn	University of Connecticut
UPR	Unfolded Protein Response
µm	mircrometer (10^{-6} m)

Word and concept definitions

Acclimatization	Ecological process by which an individual adjusts to environmental changes to survive and maintain performance. The adjustment is within the organisms' lifespan, in contrast to adaptation.
Adaptation	Evolutionary process by which a species adjusts to environmental changes to survive and maintain performance. The adjustment occurs across multiple generations, in contrast to acclimatization.
Biological replicates	Samples from biological separate samples. These combine technical and biological variability.
Biomarker	Measurable indicator of some biological state or condition.
De bruijn graph	Represents overlapping information in a set of DNA sequences and is widely used in genome and transcriptome assemblies. De bruijn graphs consist of a set of nodes representing kmers. The kmers are joined at edges seen consecutively in the input reads. New nodes and edges are generated for each new variation in the data. Polymorphism appears as a bubble within unique contigs.
Gene expression	The transcription of DNA into RNA. See transcription
Generation time	The time interval from hatching of an individual and to the hatching of its progeny.
Genotype	The genetic composition.
Hidden break	A break in the middle of the 28s rRNA. It is called hidden because under non-denaturing conditions the rRNA molecule is complete.
Homeostasis	The regulation by an organism of the chemical composition of its inner environment in order for physiological processes to proceed at optimal rates.
kmer	Words of a length k – for instance a 5mer is a word consisting of 5 letters: ATTGAC.
Phenotype	Physical characteristics based on the genotype.
Reference gene	Stably-expressed gene regardless of tissue or condition, used for normalization when calculating relative mRNA levels. Synonymous for reference gene, housekeeping gene, or internal or endogenous control.
Signal transduction	The process when a receptor detects a stimulus (e.g., ligand binding), which initiates a signaling cascade with a series of molecular events that ultimately will lead to a cellular response.
Statistical power	Ability to identify significantly expressed genes where there really is a difference. Dependent on variance, the number of replicates and sequencing depth.
Technical replicates	Samples from the biological samples that are processed separately. Accounting for technical variabilities.
Transcriptome	The complete set of RNA transcripts of an organism

Transcription	The process by which the information in a strand of DNA is copied into a new molecule of messenger RNA (mRNA).
Transcriptional profile	The transcriptome-wide expression of genes
Translation	The process by which proteins are synthesized based on the information stored in a mRNA molecule

List of figures and tables

Introduction

The growing human population is causing major changes in biotic and abiotic factors of marine environments (Bijlsma and Loeschcke, 2005; Nagelkerken and Connell, 2015). For instance, global warming is resulting in thermal stress, ocean acidification, as well as pollution and changes the chemical composition of marine environments (e.g., B. H. Hansen et al., 2010; Almén et al., 2014; Nagelkerken and Connell, 2015; Aguilera et al., 2016).

Copepods are of great ecological importance as major grazers of phytoplankton, serving as a critical link for energy transfer to higher trophic levels (Turner, 2004). Elevated temperatures and CO_2 content are causing a pelagic shift towards smaller pico – and nano-phytoplankton species that are not suitable as a food source for zooplankton, including copepods (Nagelkerken and Connell, 2015). Higher temperatures increase consumption - and metabolic rates of zooplankton and other herbivores. This is, however, not being translated into secondary production if there is no available food source (Nagelkerken and Connell, 2015). This creates an imbalance with higher trophic species, whose metabolic and foraging cost increase with temperature (Nagelkerken and Connell, 2015). It is therefore important to understand how stressors are affecting marine animals and copepods.
Besides key role as intermediaries in the marine food chain, the aquaculture industry has a growing interest in using copepods as live feed for fish larvae (Abate et al., 2015; Payne and Rippingale, 2001). The high mortality rates of first-feeding fish larvae caused by malnutrition are a major concern in fish production (reviewed in Hamre et al., 2013). Feeding copepod nauplii to the fish larvae has been shown to result in improved survival, fitness, growth and skin pigmentation in relation to traditional live feed, such as rotifers and brine shrimp (Busch et al., 2010; Conceição et al., 2010; Imsland et al., 2006; Mæhre et al., 2013; Øie et al., 2015). Despite the abundance, importance and interest of copepods in marine environments – and aquaculture industries – information on copepod physiology and genetics is limited.

This Ph.D. dissertation examines the molecular physiology of the calanoid copepod, *Acartia tonsa*. Molecular physiology can be defined as the understanding of organisms and systems at the molecular level.
This Ph.D. dissertation contributes to basic research in copepod physiology. With knowledge about the physiological molecular processes during embryonic development and in response to stress, future research can use this information for continued research in aquaculture, toxicology, general physiology and ecology.
Gene expression analysis of organisms exposed to stressful conditions gives valuable information, since changes at the transcriptional level occur before being transferred to other physiological traits (de Nadal et al., 2011). The use of gene expression can, thus, give an early indication of whether a certain condition affects an organism negatively.

The overall goal of this dissertation is to provide new information for understanding how stress affects *A. tonsa* at the transcriptional level.

Acartia tonsa

The strain of *Acartia tonsa* used in this dissertation originates from Øresund, where it was collected in 1981 (identity code: DFH.AT1, Støttrup et al., 1986). The strain was acquired by Roskilde University in the early 1990s, and has been cultivated under stable conditions ever since (personal communication, Benni W. Hansen, Roskilde University, Denmark). Decades later, the DFH.AT1 strain has been widely used in ecological, ecotoxicology, and aquaculture research (e.g., Drillet et al., 2006; Medina and Barata, 2004; Tiselius et al., 1995). Because of the well-studied nature of the DFH.AT1 strain, it has a potential of becoming model organism for studying basal physiological responses of copepods, crustaceans, and even higher-level species. Using *A. tonsa* as a model species can give a basic understanding of cellular processes (e.g., replication, transcription, and translation), physiology (e.g., reproduction, development etc.) and interactions with the surrounding environment (e.g., stress responses, circadian relationships). The strain has been cultured in a restricted population for over several hundred generations, which is assumed to result in reduced levels of genetic variation compared to wild populations. The limited variation among individuals establishes a foundation for studying stress responses at the molecular level with minimal genetic "noise" (e.g., Gasch et al., 2016). The fundamental mechanisms and processes are likely to be conserved across species; thus, the molecular stress responses of crustaceans and higher-level organisms may be explained by using *A. tonsa* (Gasch et al., 2016). The generation time for *A. tonsa* (~14-20 days at 18°C) makes it possible to investigate multiple generations, for instance in relation to epigenetics, within a limited period of time (Støttrup et al., 1986). Furthermore, the production of *A. tonsa* does not require much space, and is economically feasible (Abate et al., 2015).

A. tonsa is a robust species with wide temperature (-1 to 32°C) and salinity (S=1 to S=72) tolerances (Cervetto et al., 1999). The optimum is, however, at temperatures of ~24°C and salinities between S=15 and S=22 (Cervetto et al., 1999; Diekmann et al., 2012; Holste and Peck, 2006; Peck et al., 2015). In nature, they mainly inhabit estuaries and near-shore environments (depth: 0 – 50 m) that exhibit a large degree of environmental changes on daily basis (e.g., Diekmann et al., 2012). The restricted near-shore habitats of *A. tonsa* can be explained by the high food concentrations in estuaries and upwelled waters (Paffenhöfer and Stearns, 1988).
The species has an optimal salinity around S=17, but the DFH.AT1 strain has been adapted to higher salinities (S=27 and S=35, Jepsen et al., 2017b) corresponding to the seawater available for the laboratories at Roskilde University (Roskilde, Denmark) and the National Institute of Aquatic Resources (Lyngby, Denmark), where it is cultivated (Støttrup, 2000; Støttrup et al., 1986). In addition to the salinity of S=35, the laboratory culture conditions

at Roskilde University are temperature ~17°C, gentle aeration, dim lighting (PAR ~80µE m^{-2} s^{-1}; Vu et al., 2016) and food in excess (*Rhodomonas salina* >800 µg C L^{-1}; Berggreen et al., 1988).

The lifecycle of *A. tonsa* consists of 1 embryonic, 6 naupliar (N1-NVI) and 5 copepodite (CI-CV) stages before maturation to the adult stage (Leandro et al., 2006; Sabatini, 1990). The progression from egg to adult takes ~14 days, going through approximately one developmental stage per day at a temperature of 18°C (Leandro et al., 2006). Development of *A. tonsa* are conditionally temperature-dependent, with higher developmental rates at higher temperatures (B. W. Hansen et al., 2010). Longevity of *A. tonsa* is on average 15 days for males and 80 days for females (Mauchline, 1998; Parrish and Wilson, 1978). Survival is dependent on temperature, salinity, pH, predation and food availability (e.g., Calliari et al., 2008; Hansen et al., 2017; Mauchline, 1998). Sexually mature females produce 11-50 eggs per day, primarily spawned at night (Cervetto et al., 1993; Checkley et al., 1992; Munk and Støttrup, 1983; Parrish and Wilson, 1978; Stearns et al., 1989; Støttrup and McEvoy, 2003). This trait is, however, lost in the DFH.AT1 strain, which prefer to spawn in darkness regardless of the circadian rhythm (Jepsen et al., 2017a)

During winter and other unfavorable periods, *A. tonsa* are able to survive by producing dormant eggs (Chen and Marcus, 1997). Subitaneous eggs hatch within a few days (17°C) after spawning at favorable conditions. When the surrounding environment turns unfavorable, subitaneous eggs goes into a resting state called, quiescence (Dahms, 1995). Conditions capable of inducing quiescence include changes in temperature, salinity, oxygen and sulfide concentrations (Drillet et al., 2006; Holmstrup et al., 2006; Højgaard et al., 2008; Nielsen et al., 2006). The development of quiescent eggs will resume when favorable conditions return (Dahms, 1995). In the quiescent state, eggs are viable up to ~1 year, after which energy is depleted (Drillet et al., 2006; Holmstrup et al., 2006). Exposure to light can, however, decrease the period by which the quiescent eggs are viable (Hagemann et al., 2016).
Another type of embryonic dormancy is maternally-determined diapause. In diapause, the eggs go through a refractory phase, which can last from months to years before they hatch (Marcus, 1996). Embryonic development does not continue until the refractory phase has passed (Danks, 1987; Hairston et al., 1995; Marcus, 1996). The eggs have a higher density in relation to seawater and will, therefore, sink towards the sediment (Miller and Marcus, 1994). Dormant eggs will be deposited in the sediment, providing an egg bank for recruitment of future generations of *A. tonsa* (Hairston, 2002, 1996; Sichlau et al., 2011). Occasional disturbances in the sediment will return the dormant eggs to the water column, where they will hatch if the conditions are favorable (Hairston, 2002; Lee and McAlice, 1979).

From stress to changes in gene expression

Copepods are exposed to constant environmental alterations in nature. Estuarine copepods, like *Acartia tonsa*, are in general robust species that can adjust to wide ranges of environmental conditions. The robustness, however, has its limits. The persistence of a species or population depends upon a complex set of environmental conditions, such as temperature, pH, salinity and nutrient supply. According to Shelford's law of tolerance, each individual has definite minimum and maximum values, as well as an optimum range within the gradient of these environmental conditions (Shelford, 1911).

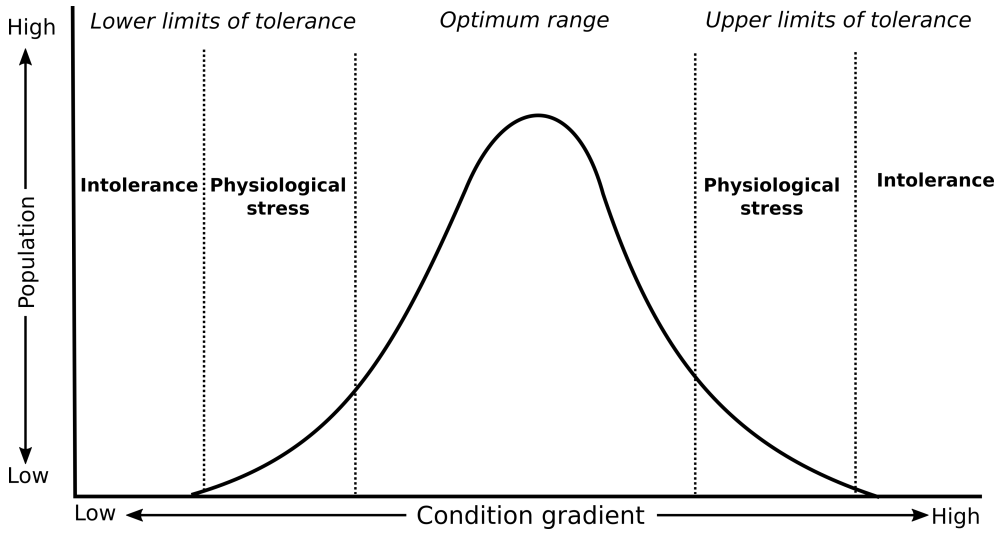

Figure 1. Illustration of Shelford's law (Shelford, 1911). An individual of a population will have an optimum range within environmental conditions were it thrives most successfully. Outside the optimum range, the individuals will experience physiological stress that will cause a decrease in the population. The further away the conditions gets from the optimum range, the more intolerance the individuals will experience and as final the population will die out. The illustration was produced using Inkscape version 0.91. Modified from Costamagno et al. (2016).

Outside the optimum range (Fig. 1), individual copepods will experience physiological stress. If the gradient of conditions continues to increase in the environment, this will have fatal consequences and may result in the local extinction of the species in that habitat (Shelford, 1911).

Organisms maintain a complex dynamic equilibrium, homeostasis, which constantly is challenged by internal and external conditions, termed stressors (Goldstein and Kopin, 2007). Stress can be defined as suboptimal conditions, which threaten the homeostasis of an organism, and thereby negatively affect viability or fitness (Goldstein and Kopin, 2007).

Stress is not only an aspect of the stressor, but also an aspect of the individual exposed to the stressor (e.g., Bijlsma and Loeschcke, 2005). The adaptive response towards stress largely depends on the organism, its current physiological state, and the environment to which the organism has been evolutionarily adapted (de Nadal et al., 2011). Even the same species may exhibit different responses toward changes when adapted to two different environments. An example is two populations of *A. tonsa* that inhabit distinct environments with contrasting carbonate system parameters; estuarine versus coastal environments (Aguilera et al., 2016). Since the estuarine system receives freshwater discharges characterized by low pH, the pressure of CO_2 is generally higher in estuarine compared to coastal environments (Aguilera et al., 2016). When incubating two populations of *A. tonsa* at the same high CO_2 pressure conditions, the estuarine copepods had higher ingestion rates compared to their coastal counterparts, suggesting that they experienced the conditions as less stressful (Aguilera et al., 2016). Expression of *HSP70* and *ferritin* were significant higher in the coastal copepods after exposure to the high pCO_2 conditions, suggesting that the estuarine population was more adapted to high CO_2 pressure, presumably due to selection from multi-generational exposure to these conditions (Aguilera et al., 2016).

Environmental stress can be defined as a response to physical changes of the environment (Bijlsma and Loeschcke, 2005). Changes in abiotic factors (e.g., temperature discharge of chemical compounds) are considered the most significant stressors regardless of whether they are naturally-occurring or man-made (Bijlsma and Loeschcke, 2005). Biotic stressors (e.g., competition, predation, parasitism) are often interrelated with abiotic stresses. For instance, an organism with reduced fitness due to an abiotic stressor may experience a higher chance of predation (e.g., Relyea, 2005). Inbreeding in populations can also cause genetic changes, making the individuals less able to adapt to environmental changes than non-inbred individuals. This can be referred to as genetic stress (Bijlsma and Loeschcke, 2005).

When facing environmental changes, an organism can either acclimatize – or adapt. Acclimatization is the progressive adjustment within the lifespan of an organism in response to changes in its environment that can be considered stressful (e.g., Hansen et al., 2010). The ability of an organism to change its phenotype in response to environmental changes is a result of phenotypic plasticity, which includes acclimatization (Badyaev, 2005; Bijlsma and Loeschcke, 2005). Adaptation is defined as genotypic and phenotypic changes over multiple generations making the organism better suited for its new environment. During natural selection of favorable heritable traits, adaptation may ultimately lead to the development of a new strain or species (i.e., evolutionary adaptation) (e.g., David et al., 2005; Drillet et al., 2008a). Genes involved in evolutionary adaptation have in general low overlap with the genes involved in phenotypic plasticity (de Nadal et al., 2011).
The concepts of adaptation and phenotypic plasticity are, however, not clearly separated. When exposed to stress, an organism will first make use of its phenotypic plasticity in order to survive the environmental changes (Kelly et al., 2012). This includes stress responses at the transcriptional level (e.g., Dayan et al., 2015; Gullian Klanian et al., 2017). If the stress

continues, selection pressure will lead to adaptation, in which the traits most suited for the new condition will be favored (Kelly et al., 2012).

Transcription

Gene expression, or more correctly transcription, is the process by which genetic information of DNA is copied and transcribed into an RNA molecule by the multi-subunit transcription enzyme, RNA polymerase (Archambault and Friesen, 1993). There are three main classes of eukaryotic RNA polymerases; each one is specialized in transcribing certain types of genes. RNA polymerase I transcribes genes encoding ribosomal RNAs (rRNAs), polymerase II transcribes messenger RNAs (mRNAs) and small nuclear RNAs (snRNAs), while RNA polymerase III is responsible for transcription of transfer RNAs (tRNAs) (Archambault and Friesen, 1993). Since genes encoding proteins are transcribed into mRNA, the focus for this research will be on RNA polymerase II.

DNA is, when not transcribed, packaged into compact structures that constitute chromosomes (Kouzarides, 2007). Before transcription can be initiated by RNA polymerase II, these chromatin structures need to loosen and the DNA double helix needs to unwind and create room for the process. Open chromatin structures facilitate transcription, while compact structures are inhibitory (Cairns, 2009). One strand of the double-stranded DNA helix is used as a template for making complementary RNA molecules. The RNA molecule is nearly identical to the genetic code of the coding DNA strand, except that Uracil bases in RNA replace Thymine bases in DNA (Alberts et al., 2008). Transcription initiates at regions called promoters located near the starting point of the genes. RNA polymerase II recognizes and interact with the promoter through a number of transcription factors. Nucleotides before the initiation site are "upstream" and marked with positive numbers for location, while nucleotides "downstream" are located after the initiation site and marked with negative numbers (Alberts et al., 2008). Many eukaryotic promoters contain sequences rich in Thymine and Adenine called the TATA box. The Adenine-Thymine interactions are easier to disrupt than Guanine-Cytosine due to two hydrogen bonds rather than three, which facilitate the unwinding of the double-stranded DNA. The TATA-box is recognized as one of the general transcription factors, allowing other transcription factors and finally RNA polymerase II to bind and interact with the gene (Alberts et al., 2008). When RNA polymerase II interacts with the promoter, nucleotides are being added to the growing RNA strand in a process called elongation. The RNA polymerase moves along the DNA strand in the 3' to 5' direction, adding the nucleotide that is complementary to each nucleotide in the DNA template. The 3' end is defined by a hydroxyl group attached to the 3rd Carbon of the sugar unit in RNA (ribose), while the 5'end is the direction of the phosphate group attached to the 5th Carbon of ribose (Alberts et al., 2008). The process will terminate when RNA polymerase II transcribes a sequence of the DNA encoding termination. The resulting pre-RNA transcript is now ready for further processing into mature mRNA (Alberts et al., 2008). During processing a 5' cap, consisting of a modified guanine nucleotide, is being added to protect the RNA transcript from degrading and aid ribosomal attachment in the cytoplasm during translation into proteins. Multiple adenine nucleotides is added to the 3'

of the RNA transcript in a poly-A tail that provides stability and aid exportation into the cytoplasm. Sections of the RNA transcript that result from non-coding DNA regions (introns) are removed in a process called splicing, and the resulting mature mRNA consists solely of protein-coding sections (exons). The pre-mRNA can undergo alternative splicing, leading to different mature mRNA molecules from the same pre-mRNA transcript (Alberts et al., 2008). The mature mRNA are exported into the cytoplasm, where they are translated into proteins by the aid of ribosomes (Alberts et al., 2008).

The complete set of mRNA present in a cell or organism at a given time is called the transcriptome. Variations of the transcriptome depends on conditions referred to here as the transcriptional profile. All cells within an organism contain DNA encoding the exact same genes. But only a fraction of these genes are expressed at any given time, causing each cell to have distinct functions based on the transcriptional profile (Alberts et al., 2008; Pierce, 2010).

Changes in mRNA levels do not directly correlate to protein amounts, but do give an indication of changes in translation when a given protein is needed by the organism (Vogel and Marcotte, 2012). The transcriptional profile is, thus, very dynamic and is tightly regulated by numerous transcription factors that either can increase or suppress the transcription of genes.

Responses to stress at the transcriptional level

Stress responses are related to changes at the cellular level. When environmental conditions exceed an optimum range, the cells of an individual must change their transcriptional patterns in order to restore homeostasis. These cellular response mechanisms are evolutionarily conserved across organisms and determine whether the organism will adapt, survive or die when exposed to stress (Grover, 2002).

Stress can be categorized as mild, chronic and acute. Acute stress, which is the focus of this dissertation, represents time-restricted and abrupt environmental alterations, to which the organism must rapidly respond in order to ensure physiological performance and survival.

Signal transduction is able to translate extracellular signals into intracellular responses, and thereby control nearly every aspect of an organism's physiology (de Nadal et al., 2011). The signal transduction pathway differs depending on the organism and type of stressor (Alberts et al., 2008). In general, an extracellular signal (e.g., ligand) interacts with a receptor, changing its conformation (Alberts et al., 2008). The conformational change of the receptor will transduce the signal (i.e., primary message) by releasing intracellular signaling molecules (i.e., secondary messengers), which initiates a phosphorylation cascade (Alberts et al., 2008). Ultimately, the signaling cascade reaches its target of action, and thereby alters expression of stress-related genes (Alberts et al., 2008). In addition to membrane-bound receptors, cells also have intracellular sensor systems that can activate signaling cascades (de Nadal et al., 2011).

An example of signal transduction is the *heat-shock protein 70kDa* (*HSP70*) response. *HSP70* is inducible in response to a number of stressors (Kregel and Sieck, 2002). During non-stressed conditions, heat-shock transcription factors (HSFs) are present in the cytoplasm of

the cells as latent monomeric molecules that are not able to bind DNA (Kregel and Sieck, 2002). When the cell senses an accumulation of damaged macro-molecules, the HSFs undergoes phosphorylation and trimerisation (Kregel and Sieck, 2002). The HSFs are then translocated to the nucleus, where they interact with at least two regulatory elements in the 5' promotor region of the *HSP70* gene and mediate *HSP70* transcription (Kregel and Sieck, 2002). When the *HSP70* proteins start to accumulate, the activity of HSF trimmers is down-regulated, as is that of heat-shock binding protein 1, which inhibits transcription (Kregel and Sieck, 2002).

Genes whose expression is induced in response to multiple stressors are considered to be universal stress response genes. The induced expression of these is generally activated by macromolecule damage, regardless of the responsible stressor (Kültz, 2005). Genes that are only induced in relation to specific stressors are, on the other hand, often involved in the reestablishment of homeostasis (Kültz, 2005).

During homeostasis, there is equilibrium between cell growth and death (Lockshin and Zakeri, 2007). The equilibrium is at risk of being shifted when the organism is exposed to stress and must respond accordingly (Fulda et al., 2010). As long as the stress is below a specific threshold, organisms are able to cope by inducing protective and recovery mechanisms (Fulda et al., 2010). If the stress exceeds the threshold or the mechanisms fail, pathways leading to cell death and removal will be activated (Fulda et al., 2010). Many of the stress responses are conserved across species and will be activated in response to multiple stressors (Kültz, 2005).

The heat-shock response was initially detected in response to heat-stress, but is actually induced in response to a wide range of stressors (Feder and Hofmann, 1999). The *heat-shock proteins* (*HSPs*) can be constitutively expressed or induced in response to environmental stressors (Feder and Hofmann, 1999). For instance *heat-shock protein 90 kDa* (*HSP90*) are constitutively expressed under non-stressed conditions and aid newly-synthesized proteins to fold correctly (Wandinger et al., 2008). *HSP70* expression, on the other hand, is induced in response to stress and protects proteins from degradation (Goloubinoff and Rios, 2007). *HSP70* can, furthermore, affect cell-death pathways by for instance releasing pro-apoptotic factors (e.g., cytochrome c) (Kennedy et al., 2014; Ravagnan et al., 2001).

Macromolecule damage is often a cellular consequence of stress. The response is the increased expression of genes encoding chaperone molecules that aid protection (Malmendal et al., 2006; Sejerkilde et al., 2003). This transient protection results in a tolerant state, where the cells exhibit increased resistance towards stress (Malmendal et al., 2006; Sejerkilde et al., 2003). This is why an organism exposed to one stressor often becomes more resistant to exposure to another one (i.e., cross resistance) (Bubliy et al., 2012; Leroy et al., 2012).

Many proteins are post-translationally processed in the endoplasmic reticulum (ER). If the production and secretion of mature proteins is no longer effective, this will cause immature proteins to accumulate and create an imbalanced homeostasis of the ER (Hetz and Papa, 2017). This ER stress will induce another cellular stress response called the unfolded protein response (UPR) (Hetz and Papa, 2017). The UPR promotes cell survival by improving the balance between protein load and folding capacity of the ER by activating a number of

factors (e.g., inositol-required protein 1, protein kinase RNA) (Hetz and Papa, 2017; Hori et al., 2006)

DNA damage caused by irradiation or environmental genotoxic agents can induce DNA damage response (e.g., Giglia-Mari et al., 2011). The DNA damage response induces repair processes aimed at restoring continuity of the DNA double strand (e.g., mismatch repair, non-homologous end-joining, homologous recombination) (e.g., Giglia-Mari et al., 2011).

Any strong stressor induces oxidative stress (Lushchak, 2011). The response towards oxidative stress is thus an important stress response in all organisms. Cells in homeostasis exist in equilibrium between oxidants and antioxidants. Any disturbance in this oxidant:antioxidant equilibrium is considered oxidative stress (Apel and Hirt, 2004). The reactive products of oxygen (i.e., reactive oxygen species, ROS), including superoxide anions ($O_2^{\bullet -}$), hydrogen peroxides (H_2O_2), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen's, peroxy radicals and nitric oxide ($NO^{\bullet}$), can cause harm to macromolecules and membranes of the cell (Apel and Hirt, 2004). Many of the ROS-metabolizing enzymes (e.g., catalase, gluthathionine peroxidase, superoxide dismutase, gluthathionine) involved in the antioxidant defense response are also commonly used as transcriptional biomarkers in copepods (Table 1). Highly-reactive OH radicals are produced via the Fenton reaction that requires metal; therefore a number of the biomarkers are used in relation to metal exposure (Table 1, Apel and Hirt, 2004). This is also why ferritin is used as biomarker, since it provides an indirect protection against ROS, by storing irons of the cells (Table 1, Harrison and Arosio, 1996; Liu and Theil, 2005). Oxidative stress often interacts with other stress response pathways, for instance, heat-shock proteins. Many heat-shock proteins are also used as biomarkers, especially *HSP70* (Table 1), due to its induction in relation to a wide range of stressors (e.g., Aruda et al., 2011; Petkeviciute et al., 2015; Rahlff et al., 2017).

Table 1. Commonly-used biomarkers for transcriptional analysis with real-time quantitative PCR in copepods. Copepod species abbreviations: *Acartia tonsa (A. tonsa); Calanus finmarchicus (C. finmarchicus); Calanus glacialis (C. glacialis); Calanus helgolandicus (C. helgolandicus); Eurytemora affinis (E. affinis)*; *Paracyclopina nana (P. nana); Pseudodiaptomus annandalei (P. annandalei); Pseudodiaptomus poplesia (P. poplesia); Tigriopus californicus (T. californicus); Tigriopus japonicus (T. japonicus);*

Biomarker	Stressors	Effect	Species	References
Aldehyde dehydrogenases (ALDHs)	Diatom toxins	Isoform dependent	*C. helgolandicus*	(Lauritano et al., 2016, 2011a)
Glutathione S-transferases (GSTs)	Endocrine disrupting chemicals	Concentration and stressor dependent	*T. japonicus*	(Lee et al., 2006)
	Toxic diatoms and dinoflagellates	Time and/or isoform dependent	*C. helgolandicus, C. finmarchicus*	(Lauritano et al., 2016, 2011a; Roncalli et al., 2016b)
	Heavy metals (Cu, Mn, Ag, As, Cd)	Time, metal-type and isoform dependent	*T. japonicus*	(K.-W. Lee et al., 2008b; Lee et al., 2007)
	Naphthalene	Elevated expression	*C. finmarchicus*	(Hansen et al., 2008)
		Elevated expression (peak)	*P. poplesia*	(Zhuang et al., 2017)
	Hydrogen peroxide	Time dependent	*T. japonicus*	(Lee et al., 2007)
	Pyrene	Time and isoform dependent	*P. poplesia*	(Zhuang et al., 2017)
	Water-soluble fractions and water accommodated fractions of crude oil, oil droplets	Concentration - and time dependent	*C. finmarchicus, C. glacialis*	(Hansen et al., 2011, 2009)
	Diethanolamine	Concentration and time dependent	*C. finmarchicus*	(B. H. Hansen et al., 2010)
	Triphenyltin chloride	Down regulation	*T. japonicus*	(Yi et al., 2014)
Cytochromes P450 (CYPs)	Diatom toxins	Isoform dependent	*C. helgolandicus*	(Lauritano et al., 2016, 2011a)
	Naphthalene	Concentration dependent down regulation	*C. finmarchicus*	(Hansen et al., 2008)
	Diethanolamine	Down regulation	*C. finmarchicus*	(B. H. Hansen et al., 2010)
	Water-soluble fractions of crude oil and oil droplets	Stressor dependent	*C. finmarchicus*	(Hansen et al., 2009)
	Polycyclic aromatic hydrocarbons, water accommodated fractions of crude oil	Elevated expression	*P. nana*	(Han et al., 2015a)

Table 1. *Continued from previous page.*

Biomarker	Stressors	Effect	Species	References
Glutathione reductase (GR)	Salinity	Elevated for high salinities, down-regulated for low	*T. japonicus*	(Seo et al., 2006a)
	Heavy metals (Cu, Mn)	Elevated expression	*T. japonicus*	(Weaver et al., 2016)
	Hydrogen peroxide	Concentration and time dependent	*T. japonicus*	(Seo et al., 2006a)
Superoxide dismutases (SODs)	Heavy metals (Cu, Zn, Ag)	Elevated expression at high concentrations	*T. japonicus*	(Kim et al., 2011)
	Endocrine disrupting chemicals	Concentration and stressor dependent	*T. japonicus*	(Kim et al., 2011)
	Naphthalene	No significant change	*C. finmarchicus*	(Hansen et al., 2008)
	Toxic diatoms	No significant change	*C. helgolandicus*	(Lauritano et al., 2011a)
		Down regulation	*C. helgolandicus*	(Lauritano et al., 2016)
	Nickel	Elevated expression (dose response)	*P. annandalei*	(Jiang et al., 2013)
Catalases (CATs)	Naphthalene	No significant change	*C. finmarchicus*	(Hansen et al., 2008)
	Toxic diatoms	No significant change	*C. helgolandicus*	(Lauritano et al., 2011a)
		Elevated expression	*C. helgolandicus*	(Lauritano et al., 2016)
Glutathione Peroxidases (GPxs)	Pyrene	No significant change	*P. poplesia*	(Zhuang et al., 2017)
	Naphthalene		*P. poplesia*	(Zhuang et al., 2017)
Heat-shock protein 10, 20, 21, 22, 40, 60, 94 and 105 kDa	Temperature	No significant change	*T. japonicus*	(Rhee et al., 2009)
		Elevated expression for high -, down regulation for low temperatures	*T. japonicus*	(Seo et al., 2006b)
	Salinity	No significant change	*T. japonicus*	(Seo et al., 2006b)
	Endocrine disrupter chemicals	Stressor dependent	*T. japonicus*	(Seo et al., 2006c)
	Handling	Elevated expression	*C. finmarchicus*	(Aruda et al., 2011)
	Diapause	Elevated expression	*C. finmarchicus*	(Aruda et al., 2011)
	Toxic diatoms	Down regulation	*C. helgolandicus*	(Lauritano et al., 2011a)
Glucose-Regulated Protein, 78kDa	Temperature	Elevated expression	*E. affinis*	(Xuereb et al., 2012)
	Salinity shock	Elevated expression	*E. affinis*	(Xuereb et al., 2012)
Vitellogenin	Heavy metals (Cd, Ag, As, Cu)	Metal dependent	*T. japonicus*	(K.-W. Lee et al., 2008a)
		Elevated expression	*P. nana*	(Hwang et al., 2010b)
Ubiquitin	Naphthalene	No significant change	*C. finmarchicus*	(Hansen et al., 2008)
	Diethanolamine	Concentration dependent	*C. finmarchicus*	(Hansen et al., 2011)

Table 1. *Continued from previous page.*

Biomarker	Stressors	Effect	Species	References
Heat-shock protein 70kDa (HSP70)	Heat-shock / increasing temperature	Elevated expression (acclimatization result in lower expression)	*A. tonsa*	(Rahlff et al., 2017)
			A. tonsa	(Petkeviciute et al., 2015)
			E. affinis	(Rahlff et al., 2017)
			T. japonicus	(Rhee et al., 2009)
			T. californicus	(Chan et al., 2014)
			C. finmarchicus	(Voznesensky et al., 2004)
		No significance	*C. finmarchicus*	(Smolina et al., 2015)
	Handling	Elevated expression	*C. finmarchicus*	(Aruda et al., 2011; Rahlff et al., 2017)
			E. affinis	(Rahlff et al., 2017)
	Salinity	Concentration dependent, but in general elevated expression outside acclimatization range	*A. tonsa*	(Petkeviciute et al., 2015)
	UV-radiation	Elevated expression of the constitutively expressed *HSP70*, no significance for the inducible form of *HSP70*	*A. tonsa*	(Tartarotti and Torres, 2009)
	Crowding	No significance	*A. tonsa*	(Nilsson et al., 2017)
	Quiescence	Time dependent	*A. tonsa*	(Nilsson et al., 2013)
	CO_2 pressure	Acclimatization dependent (elevated expression for coastal copepods compared to estuarine copepods)	*A. tonsa*	(Aguilera et al., 2016)
	Shallow active vs. deep diapausing copepods	Elevated expression for active copepods in shallow waters	*C. finmarchicus*	(Aruda et al., 2011)
	Naphthalene	No significant change	*C. finmarchicus*	(Hansen et al., 2008)
	Toxic diatoms	Elevated expression	*C. helgolandicus*	(Lauritano et al., 2016)
		No significant change	*C. helgolandicus*	(Lauritano et al., 2011a)
	Heavy metals (Cu, Ag, Zn)	Elevated expression	*T. japonicus*	(Rhee et al., 2009)
	Endocrine disrupting chemicals	Concentration and /or stressor dependent	*T. japonicus*	(Rhee et al., 2009; Yi et al., 2014)
Glutathione synthase	Toxic diatoms	No significant change	*C. helgolandicus*	(Lauritano et al., 2011a)
	Diethanolamine	Concentration dependent	*C. finmarchicus*	(Hansen et al., 2011)

Table 1. *Continued from previous page.*

Biomarker	Stressors	Effect	Species	References
Heat-shock protein 90kDa (HSP90)	Heat shock / increasing temperature	No significant change	*T. japonicus*	(Rhee et al., 2009)
		Elevated expression	*E. affinis*	(Xuereb et al., 2012)
		Elevated expression	*A. tonsa*	(Petkeviciute et al., 2015)
	Salinity shock	Concentration dependent, elevated expression outside acclimatization range	*A. tonsa*	(Petkeviciute et al., 2015)
			E. affinis	(Xuereb et al., 2012)
	Naphthalene	No significant change	*C. finmarchicus*	(Hansen et al., 2008)
	Crowding	No significant change	*A. tonsa*	(Nilsson et al., 2017)
Cell cycle and apoptosis regulatory 1 protein	Toxic diatoms	No significant change	*C. helgolandicus*	(Lauritano et al., 2011a)
p53 tumor suppressor protein	Endocrine disrupting chemicals	Elevated expression	*T. japonicus*	(Hwang et al., 2010a)
Retinoid X receptor	Triphenyltin chloride	Down regulation	*T. japonicus*	(Yi et al., 2014)
Gamma-glutamylcysteine synthase	Diethanolamine	Concentration dependent	*C. finmarchicus*	(Hansen et al., 2011)
Peroxiredoxin-6	Naphthalene	Elevated expression	*P. poplesia*	(Zhuang et al., 2017)
Ras-related C3 botulinum toxin substrate 1	Naphthalene	Elevated expression	*P. poplesia*	(Zhuang et al., 2017)
Methylmalonate-semialdehyde dehydrogenase	Pyrene	Elevated expression	*P. poplesia*	(Zhuang et al., 2017)
Ribosomal protein L13	Nickel	Elevated expression	*P. annandalei*	(Jiang et al., 2013)
Separase	Nickel	Elevated expression	*P. annandalei*	(Jiang et al., 2013)
Myohemerythrin-1	Nickel	Elevated expression	*P. annandalei*	(Jiang et al., 2013)
Ferritin	Crowding	No significance	*A. tonsa*	(Nilsson et al., 2017)
	Quiescence	Time dependent peaks	*A. tonsa*	(Nilsson et al., 2013)
	Epibiont infestation	Elevated expression	*A. tonsa*	(Petkeviciute et al., 2015)
	CO_2 pressure	Acclimatization dependent (elevated expression for coastal copepods compared to estuarine copepods)	*A. tonsa*	(Aguilera et al., 2016)
	Nickel	Elevated expression	*P. annandalei*	(Jiang et al., 2013)
Inhibitor of apoptosis protein	Toxic diatoms	Down regulation	*C. helgolandicus*	(Lauritano et al., 2011a)
Cellular apoptosis susceptibility protein	Toxic diatoms	Down regulation	*C. helgolandicus*	(Lauritano et al., 2016)
Tubulins	Toxic diatoms	Down regulation	*C. helgolandicus*	(Lauritano et al., 2011b)
	Nickel	Elevated expression	*T. japonicus*	(Jiang et al., 2013)

Table 1. *Continued from previous page.*

Biomarker	Stressors	Effect	Species	References
COI	Copper	Elevated expression	*T. japonicus*	(Weaver et al., 2016)
Toll-like receptor	Increasing temperature	Elevated expression	*T. californicus*	(Chan et al., 2014)
Glutamate Dehydrogenase	Increasing temperature	Elevated expression, time dependent	*C. finmarchicus*	(Smolina et al., 2015)
Nucleosome Assembly Protein 1	Increasing temperature	Elevated expression, time dependent	*C. finmarchicus*	(Smolina et al., 2015)
Ribosomal Protein S11	Increasing temperature	No significant change	*C. finmarchicus*	(Smolina et al., 2015)
DnaJ homolog	Increasing temperature	No significant change	*C. finmarchicus*	(Smolina et al., 2015)
Corticotropin Releasing Hormone Binding Protein	Salinity	Elevated expression	*T. japonicus*	(K.-W. Lee et al., 2008b)
	Salinity	No significant change	*T. californicus*	(Willett and S. Burton, 2003)
Glutamate dehydrogenase	Salinity	No significant change	*T. californicus*	(Willett and S. Burton, 2003)
Delta-1 pyrroline-5-carboxylase reductase	Salinity	No significant change	*T. californicus*	(Willett and S. Burton, 2003)
Delta-pyrroline-5-carboxylate synthase	Salinity	No significant change	*T. californicus*	(Willett and S. Burton, 2003)

When a stressor exceeds the capacity of an organism's defense mechanisms, ellular stress responses are replaced with pathways leading to cell death and removal (Fulda et al., 2010). Apoptosis, the classic perception of cell death, is a series of biochemical events resulting in changes in cell morphology that ultimately destroy the cell (Elmore, 2007). Apoptosis can be initiated through intrinsic and extrinsic pathways (Elmore, 2007). In the intrinsic pathway, the cell senses stress and kills itself, while other cells signal the cell to kill itself in the extrinsic pathway (Elmore, 2007). Both pathways activate caspases, which are protein-degrading enzymes (e.g., proteases) (Elmore, 2007). In addition to degrading the proteins of the cell, the caspases also neutralize the effects of inhibitors of apoptosis proteins (IAP) (Shi, 2002). In contrast to apoptosis, necrosis a less-regulated and more-traumatic form of cell death that results from acute of cellular injuries (Fulda et al., 2010).

Autophagic cell death is another terminal pathway, in which the cellular components undergo degradation. The cellular components are engulfed and transported to the lysosome by a double-membraned vesicle, the autophagosome (Gozuacik and Kimchi, 2007). When the autophagosome fuses with the lysosome, the contents will be degraded and recycled (Gozuacik and Kimchi, 2007).

Besides the cellular stress-response genes, a number of biomarkers related to cell death are also commonly used as biomarkers in copepods (Table 1). Especially genes related to apoptosis, such as *ubiquitin* and *IAP*, are being used (Table 1).

Stress responses are typically transient, meaning that gene expression after a period of time will reach a steady-state close to the level found in non-stressed cells (López-Maury et al., 2008).

When stress-related genes are up-regulated, energy-consuming processes, often growth-related, will be down-regulated, in order to reallocate energy to survival mechanisms (de Nadal et al., 2011; López-Maury et al., 2008). The down-regulation of growth-related genes, such as *vitellogenin* (Table 1), are therefore also useful biomarkers in relation to stress.

Presentation of the Scientific papers

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MANUSCRIPT I: Copepod swimming behavior, respiration, and expression of stress-related genes in response to high stocking densities

Producing *Acartia tonsa* in high densities is a major challenge. The restriction in production has been hypothesized to be caused by stressors associated with crowding of the individuals.

We evaluated responses of *A. tonsa* to high-density conditions (up to 10,000 ind. L^{-1}) up to 12 hrs of exposure, including assessments of swimming behavior, respiration, and transcriptional biomarkers. The results indicated that none of the elevated densities caused any changes in any of the indicators assessed in this study.

This paper was published in the journal *Aquaculture Reports* on March 2, 2017. I am the first author on this paper; I was primarily involved in analysis of gene expression, but also in several other aspects of the study.

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Copepod swimming behavior, respiration, and expression of stress-related genes in response to high stocking densities

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ABSTRACT

Using copepod nauplii as live feed in aquaculture hatcheries could solve high mortality rates of first-feeding fish larvae due to malnutrition. However, implementing the use of copepod nauplii on an intensive production scale requires a stable production at preferably high densities, which is problematic for calanoid copepod species like *Acartia tonsa*. In the present study, we evaluated the response of copepods experiencing stress under high-density conditions by assessing the acute stress level of *A. tonsa*. Control density was at 100 ind. L^{-1} while the treatments were increased stepwise up to 10,000 ind. L^{-1}. Three biological/physiological end-points were studied: swimming behavior, respiration rate and expression level of stress-related genes.

None of the elevated densities caused any significant change in swimming behavior, respiration rate or gene expression level. This study suggests that adults of *A. tonsa* do not exhibit any measurable acute stress response when exposed to high culture densities for 12 h.

1. Introduction

One major challenge in fish production is the high mortality rates of first-feeding fish larvae caused by malnutrition (reviewed in Hamre et al., 2013). Feeding copepod nauplii to fish larvae have shown to decrease the effect of malnutrition by improving survival, fitness, growth and skin pigmentation compared to traditional live feed like brine shrimps and rotifers (e.g. Øie et al., 2015). These benefits have resulted in a growing interest in implementing copepods as live feed on an intensive scale (Abate et al., 2015; Payne and Rippingale, 2001). In order to support the production of fish larvae, a stable copepod production at preferable high densities is required. For instance, a prototype of an intensive recirculation aquaculture system for production of *Acartia tonsa* is currently present at Roskilde University (Denmark) (described in Abate et al., 2015). Similar cultivation systems are available for other calanoid copepod species (e.g. Carotenuto et al., 2012). High copepod densities are, however, considered a challenge when implementing copepods as a live feed on an intensive scale (Ajiboye et al., 2011; Drillet et al., 2011).

High copepod densities can result in different types of stressors including limited food resources, oxygen depletion, accumulation of metabolic products and the physical interaction with other individuals (Jepsen et al., 2015; Ozaki et al., 2010; Støttrup and Norsker, 1997). The negative effect of the individual and multiple stressors in high-density conditions can explain why copepods are difficult to raise in dense cultures (e.g. Støttrup and McEvoy 2003; Jepsen et al., 2007). How these stressors are inter-related and how they affect each other is not well understood. Nevertheless, an optimal density at which a copepod population has its optimal "output" has been demonstrated as proposed and modeled by Drillet and Lombard (2013) and Drillet et al. (2014a,b).

Culture densities ranging from 50 to 600 mature *A. tonsa* L^{-1} have been reported without having any general negative effects on the copepods (Jepsen et al., 2007; Ogle, 1979; Peck and Holste, 2006; Støttrup et al., 1986). For adult densities ranging from 100–250 ind. L^{-1}, *A. tonsa* have been reported to have an optimal egg production of 25–39 eggs $female^{-1}$ day^{-1} (e.g. Franco et al., 2017). High-density studies of adult *A. tonsa* up to 6000 ind. L^{-1} have been reported with negative responses on adult survival,

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fecundity and developmental time as well as cannibalism of eggs and nauplii (e.g. Drillet et al., 2014a,b; Franco et al., 2017). Despite of copepods potential economic value in aquaculture industries, knowledge about their biology and physiology in relation to high densities is, however, limited (Abate et al., 2015; Drillet et al., 2011).

So far, the mechanisms underlying the difficulties of rearing high-density copepod cultures are still unknown. In the present study, we are challenging the perception of adult copepods being stressed at high-density conditions up to 10,000 ind. L^{-1} by examining three physiological end-points: swimming behavior, respiration rate and gene expression analysis of stress-related genes (*ferritin*, *hsp70*, *hsp90*).

We want to examine the following:

- If high-density conditions up to 10,000 ind. L^{-1} are stressful for individuals of *A. tonsa*, will we find a change in swimming behavior compared to lower densities (100 ind. L^{-1})?
- Will there be changes in respiration rates over densities indicating a stress response?
- Will three chosen transcriptional stress biomarkers (*ferritin*, *hsp70*, *hsp90*) exhibit changes in gene expression between high – and low-density conditions?

2. Materials and methods

2.1. Copepod cultures

The culture strain of *A. tonsa* originated from Øresund (N 56°/E 12°; Denmark) where the animals were collected by the National Institute of Aquatic Resources, Danish Technical University (Denmark) in 1981 (identity code: DFH.AT1) (Støttrup et al., 1986). The strain has been cultivated for 36 years under constant salinity, temperature and light conditions (0.2 μm filtered seawater, salinity 30–32 psu, 17 °C, oxygen >60%, dim light). The copepods have been kept at the same conditions for 25 years in 60 L polyethene tanks at Roskilde University (Denmark) and fed the mono-algae, *Rhodomonas salina,* in excess (>800 μg C L^{-1}; Berggreen et al., 1988). *R. salina* was cultivated in 2 L round-bottom flasks diluted daily with Guillard's (F/2) medium (Guillard and Ryther, 1962). Algae-cultivation took place under a stable temperature (17 °C) with constant CO_2 supply and light (PAR ~80 μE m^{-2} s^{-1}).

For the three experiments conducted, a mixture of CIV-CV copepodites and mature individuals of *A. tonsa*, measured under a dissection microscope (Olympus SZ 40, Olympus opticals (Europa) GmBH, Hamburg, Germany) at 40× magnification (prosome length: 780 ± 70 μm/675 ± 85 μm, n = 250, female:male ratio: ~1:1, referred to as adults) from 24 to 26 days old stocks (grown from cold-stored eggs), were being used. The stock densities ranged from ~ 500–1000 ind. L^{-1}. The density of 100 adults L^{-1} was used to represent very low-density conditions, while 5000 and 10,000 ind L^{-1} represented high culture densities. In addition to these densities, we included densities of 1250 ind L^{-1} for the swimming behavior and 500, 1250 and 2500 adults L^{-1} in the respiration experiment to ensure that, despite technical limitations, we could monitor response for lower densities. Incubation periods of 1 h, 8 h and 12 h were applied for all three experiments.

2.2. Swimming behavior

Besides densities of 100, 5000 and 10,000 ind. L^{-1} an additional density of 1250 ind. L^{-1} were used as a low-density treatment. In preliminary studies (not shown here), we found that 100 ind. L^{-1} in some cases is difficult to record since there are not enough animals for capture. To avoid this, we chose 1250 ind. L^{-1} as the lowest possible density at which we could get sufficient recordings of the animals for analysis. After being transferred gently with a 400 μm mesh to triplicate 250 mL tissue-culture flasks, the copepods were left to rest in complete darkness for 15 min in order to calm the convective water movement. Each replicate was recorded at 25 fps by a monochrome USB3 digital camera (model DMK23UM021; The Imaging Source Europe GmbH, Bremen, Germany) after 1 h, 8 h and 12 h. The camera was mounted with a 105 mm Nikkon lens in a setup described in Hansen et al. (2010b). In brief, light was provided by an infrared diode collimated by a Fresnel lens directing the light beams directly into the camera, which gave very high contrast and allowed optimal apparatus setting of the lens. With this setup, the entire depth of the tissue flask was visible. The videos were stored directly on a PC as Quicktime movies, which subsequently were analyzed using the motion analysis software Labtrack 4™ (BioRAS, Kvistgaard, Denmark).

The motion analysis extracted the calibrated vertical and horizontal positions of the copepods from the Quicktime movie while keeping track of the time of multiple copepods simultaneously. The motion analyses were conducted at time steps corresponding roughly to the time step where the copepod had moved at least one body length in the Quicktime movie.

The digitalized motility patterns were subsequently analyzed for the characteristic motility descriptors following Visser and Kiørboe (2006). An idealized swim path is shown on Fig. 1A. Visser and Kiørboe (2006) suggested that the net displacement l traveled by a copepod can be described by the diffusive random walk model of Taylor (1921).

$$l = \sqrt{2v^2\tau\left(t - \tau\left(1 - e^{-t/\tau}\right)\right)} \quad (1)$$

Eq. (1) estimates the characteristic parameters of random walk behavior. The random walk model describes a particle (here a copepod) as moving in an initially ballistic path at the velocity v over time t. As the copepod shift directions over time, the motility becomes more convoluted and diffusive (Fig. 1A). τ is equal to the time point where the motility changes from ballistic to diffusive and displays similar characteristics as the tumble frequency.

The net displacement l (cm) for each time step was determined as the root mean squared (RMS) for each time step for all individuals recorded for each video recording. In an observational system with two dimensions (horizontal and vertical planes), diffusion rate (D) is given by Eq. (2) (Berg, 1983):

$$D = \frac{v^2\tau}{2} \quad (2)$$

Thus Eq. (2) can be substituted into Eq. (1):

$$l = \sqrt{4D\left(t - \tau\left(1 - e^{-t/\tau}\right)\right)} \quad (3)$$

We then fitted the net displacement by time to Eq. (3) for each treatment (See example in Fig. 1B) by non-linear regression (SAS 9.4 ™ NLIN library), to estimate D (diffusion rate) and τ (tumbling frequency, s^{-1}) of the densities of 100, 1250, 5000 and 10,000 copepods L^{-1}.

2.3. Respiration

Individuals of *A. tonsa* were initially incubated at densities 100, 5000 and 10,000 ind. L^{-1}, but since the average O_2 decline at the lowest density of 100 ind. L^{-1} was not statistically significant ($t = -2.47$, $df = 5$, $p > 0.05$), we repeated the setup with additional densities of 500, 1250, and 2500 ind. L^{-1} to ensure that significant O_2 declines could also be measured at low-density conditions. The copepods were kept in 25 mL gas-tight glass bottles containing seawater (0.2 μm filtered, 30–32 psu, air-equilibrated). Five

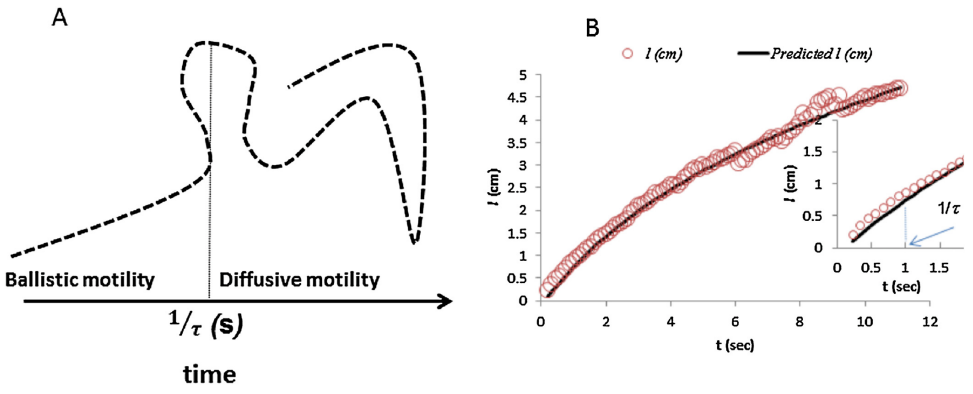

Fig. 1. Panel A shows the conceptual swimming path of *Acartia tonsa*. The animal is initially moving ballistically in a straight path (left hand side of figure). As time passes (marked by arrow shaped x-axis), the behavior changes from ballistic to diffusive. The dotted vertical line denote the time ($1/\tau$), where the *A. tonsa* jumps, and the swimming path becomes convoluted, and the motility changes to diffusive (right hand side of the dotted line on Fig. 1A). Panel B show an example of a treatment where *l* ( ) is calculated and fitted to Eq. (3) (full black curve line). The small insert shows where the statistical software (SAS 9.4) estimated the transition from ballistic to diffusive.

replicate bottles were prepared for each animal density and for control incubations without animals. The bottles were mounted on a plankton wheel that was submerged in a temperature-controlled water bath (17 ± 0.5 °C) and rotated at 10 rpm. Oxygen concentration was monitored every 60–120 min using O_2-sensitive planar optodes (SensorSpot, Pyroscience, Germany) glued to the inside of the bottles and interrogated with an optical O_2 meter (FireSting, Pyroscience, Germany). Before and after the experiment, each SensorSpot was individually calibrated in seawater (30–32 psu, temperature 17 °C) at O_2 concentrations corresponding to 100 and 0% air saturation (i.e., 251.5 and 0 μmol O_2 L^{-1}). Individual-specific respiration rates were calculated from the linear decrease in O_2 concentration during the first 6–8.5 h of the experimental incubation, corrected for the minute changes in O_2 concentration observed in the control bottles. Biomass-specific respiration rates were calculated from individual-specific respiration rates by using the relationship between prosome length L (μm) and organic carbon content B (ng C) in *A. tonsa* determined by Berggreen et al. (1988): $B = 1.11 \times 10^{-5} L^{2.92}$.

2.4. Gene expression

Quadruplicate flasks with densities of 100, 5000 and 10,000 ind. L^{-1} were prepared for each of the following periods: 1, 8 and 12 h of incubation. From each incubation flask, 25 copepods were transferred to 1 mL RNAlater® (Sigma–Aldrich, USA). This resulted in 4 biological replicates for each treatment and incubation period, consisting each of 25 animals. The samples were stored at −80 °C until RNA extraction.

RNA extraction was performed with the RNeasy Mini kit (Qiagen, Germany). The frozen samples were thawed on ice and excess RNAlater® (Sigma–Aldrich, USA) was removed. The animals were homogenized using a disposable micro-pestle in lysis-buffer from the RNeasy Mini kit (Qiagen, Germany). The following RNA extraction and purification were performed according to the manufacturer's protocol. The RNA samples were treated with DNAse I (Fermentas, USA) to remove genomic DNA.

RNA concentrations and purity were measured using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific Inc., USA). RNA integrity was checked, on a denaturing 1% agarose gel stained with ethidium bromide. An aliquot of 100 ng of total RNA from each sample was reverse transcribed to complementary first-strand DNA (cDNA) with TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, USA) using oligo$(dT)_{16}$. The reverse transcription polymerase chain reactions (PCR) were performed as described in the manufacturer's protocol.

Real-time quantitative PCR was done in 12.5 μL reactions with 6.25 μL Brilliant® II Master Mix (Sigma-Aldrich, USA), 0.5 μL 1.0 mM forward primer, 0.5 μL 1.0 mM reverse primer, 2.75 μL of RNase/DNase free water and 2.25 μL of the first strand cDNA template. Primers for *ferritin*, *hsp70* and *β-actin* were used as described in Nilsson et al. (2013) and primers for *hsp90* and *efa-1α* as described in Petkeviciute et al. (2015). The reactions were run on Stratagene Mx3005P (AH Diagnostics, Aarhus V, Denmark) real-time thermal 40 cycler as follows: 95 °C/15 min, 40 cycles [95 °C/30 s], 60 °C/30 s. Each of the biological replicates was run as technical triplicates during the analysis. PCR amplification efficiencies were 95–97%.

Gene expression levels were normalized by using *β-actin* as reference gene and the $2^{-\Delta\Delta Ct}$ method to estimate relative mRNA levels (Livak and Schmittgen, 2001). Two reference genes, *β-actin*, and *EFA-1a*, were initially used but only β-actin exhibited stable expression during the course of the experiment and was therefore chosen for normalization.

2.5. Statistical analysis

The estimated diffusion rates and tumbling frequencies were compared by density by an unbalanced ANOVA using a GLM model (SAS™ software GLM library), to determine whether diffusion rate or tumbling frequency were significantly different between density and time. We used density (treatment) and time as class variable in the statistical model. Time was used as a class variable in the model because we have no reason to hypothesize any linear response in the tested variable to time. The triplicate observations on the 100 ind. L^{-1} were pooled due to very few observations at the low concentration. To that end, we were unable to record any animals in the 100 ind. L^{-1} treatments. We did not do any time step recordings at 1250 ind. L^{-1}. We used an unbalanced ANOVA, to circumvent the different number of replicates and missing time steps among treatments. The ANOVA was followed by a pairwise post hoc test

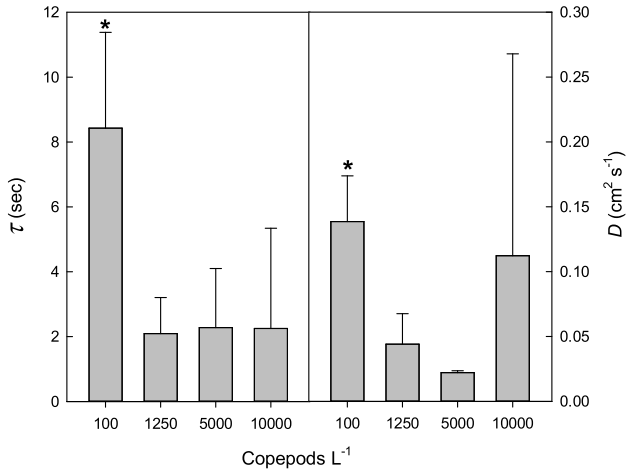

Fig. 2. The characteristic swimming path parameters by the calanoid copepod *Acartia tonsa* (tumbling frequency, τ and the diffusion rate, *D*) derived by equation 3 after one-hour incubation for the four treatments. Error bars are the standard deviation and statistical difference is marked by an asterisk (*).

Table 1
Fit parameters of equation 3 for calculating the net displacement, *l* (cm): $l = \sqrt{4D\left(t - \tau\left(1 - e^{-t/\tau}\right)\right)}$ whereas *D*, is the diffusion rate ($cm^2\ s.^{-1}$), t is the time (s) and τ is tumbling frequency (s^{-1}). Data are fit values ± standard deviations obtained by the non-linear fitting of equation 3 for each of the three replicates for each density and incubation time. Note that due to the low number of observations in the 100 *Acartia tonsa* copepods L^{-1}, the standard deviation has been replaced by standard errors derived from the non-linear fit (marked by an *).

Copepods L^{-1}	time (h)	D ($cm^2\ s^{-1}$)	τ (s)
100	1	0.140 ± 0.035*	8.43 ± 2.95*
100	8	0.049 ± 0.002	5.19 ± 0.42
1250	1	0.044 ± 0.023	2.09 ± 1.11
5000	1	0.022 ± 0.001	2.28 ± 1.82
5000	8	0.017 ± 0.001	1.76 ± 1.44
5000	12	0.027 ± 0.001	1.95 ± 0.61
10000	1	0.110 ± 0.155	2.25 ± 3.10
10000	8	0.024 ± 0.002	1.36 ± 0.24
10000	12	0.019 ± 0.005	0.73 ± 0.45

(Tukey's test) to identify statistical differences among concentrations.

A one-way ANOVA was used to test for significant differences in respiration among the densities.

Since the number of biological replicates (N = 4) was limited in the gene expression analysis, the distribution of the data could not be estimated. Furthermore, low replicate numbers usually result in that the variance among groups that are not well represented. Therefore a multiple *t*-test without assuming equal standard deviation was used to determine if treatments were significantly different with Holm-Šidák step down comparisons (alpha of 5%) (Ludbrook, 1998).

3. Results

3.1. Swimming behavior

Table 1 represents the parameters of equation 3 containing the densities (100, 1250, 5000 and 10,000 ind. L^{-1}), the incubation times (1, 8 and 12 h), the estimated diffusion rates, D, and tumbling frequencies, τ. The diffusion rate is in general terms the volume that the copepods "explore" per second. The apparent decrease in diffusion rate with increasing densities means that the copepods appear to cover a smaller volume of water. The tumbling frequency (τ.) also appears to decrease over time, meaning that the copepods jump more over time at all densities. The ANOVA followed by the Tukey test showed that the diffusion rate and the tumbling frequency of 100 copepods L^{-1} were significantly different ($p < 0.05$) than for the densities of 1250, 5000 and 10,000 copepods L^{-1} after one hour (Fig. 1B, Table 1 and Fig. 2). The three other treatments, excluding the 100 copepods L^{-1}, of 1200, 5000 and 10,000 copepods L^{-1}, however, showed no statistical significant difference in diffusion rate over time (mean ± SD: 0.038 $cm^2\ s^{-1}$ ± 0.027 $cm^2\ s^{-1}$) or in tumbling frequency (mean ± SD: 1.78 s^{-1} ± 1.25 s^{-1}, Table 1).

3.2. Respiration

Biomass-specific respiration rates (nmol O_2 μmol C^{-1} h^{-1}) versus densities (500, 1250, 2500, 5000 and 10,000 ind. L^{-1}) are shown in Fig. 3. The average respiration rate appears to slightly increase with increasing copepod density, which is then followed by a slight decrease at even higher densities. One-way ANOVA revealed, however, that the respiration rate did not vary significantly with copepod density ($df = 4.20$; $F = 1.066$; $p = 0.40$).

3.3. Gene expression

Relative mRNA levels for copepod densities of 100, 5000 and 10,000 ind. L^{-1} at 1, 8 and 12 h of incubation are given in Fig. 4. Comparison of the relative gene expression levels of *ferritin*, *hsp70* and *hsp90* between densities of 100 ind. L^{-1} did not vary over time (Holm-Šidák method, $p > 0.05$). These densities represent low culture density and were therefore used as controls for statistical comparisons. None of the higher densities exhibited any significant changes in relative mRNA levels of *ferritin*, *hsp70* or *hsp90* for any of the incubation times (Holm-Šidák method, $p > 0.05$, Fig. 4).

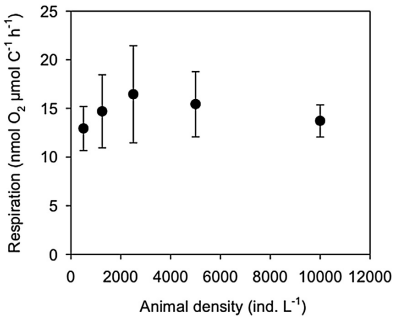

Fig. 3. Biomass-specific respiration of the calanoid copepod *Acartia tonsa* plotted against density (ind. L^{-1}). There was no significant change in respiration rate over densities (one-way ANOVA, df = 4.20; F = 1.066; p = 0.40). Error bars represents standard deviation (n = 5).

4. Discussion

Calanoid copepods, including *A. tonsa*, are suspension feeders and require large volumes of water. Maximum culture densities of *A. tonsa* usually range from 100 to 2,000 ind. L^{-1} (Støttrup, 2006 and references herin). Actual culture-wide densities can, however, be difficult to estimate and only few studies are available on *A. tonsa* densities from eggs to adults (e.g. Franco et al., 2017).

We found that individuals of *A. tonsa* did not exhibit stress response at the behavioral, respiratory or at gene expression level in relation to high-density conditions (10,000 ind. L^{-1}) during 12 h. Furthermore, mortality at densities of up to 20,000 ind. L^{-1} has been shown to be below 5% at incubation times of 48 h for the same copepod stocks as used in the present study (personal communication, Mads Kærhus Olufsen, Roskilde University). This corresponds to other studies with constant daily mortality rates of 5 – 9.75% for densities up to 5000 ind. L^{-1} (Drillet et al., 2014b; Medina and Barata, 2004; Støttrup et al., 1986). A recent study by Franco et al. (2017), using the same strain of *A. tonsa* as in the present study, did however observe a decrease in survival (2-fold) for densities above 1000 ind. L^{-1}.

The >30 year old copepod strain (Støttrup et al., 1986) is well described in the literature. It is an advantage that the same culture baseline applies for many studies. Furthermore, copepods for use as live feed in aquaculture hatcheries would probably end up being cultivated for multiple generations. When starting copepod cultures, high densities are obtained the first few days followed by a decline and stabilization at a lower level (Støttrup et al., 1986; and references herein). So far, there are no clear answers on why *A. tonsa* is difficult to rear at high densities long-term. The present study focused on acute stress responses up to 12 h as a result of high-density conditions and did not investigate the long term responses.

Planktonic organisms cover a wider behavioral spectrum. *A. tonsa* "drift" motionless while searching for food. The drifting is frequently interrupted by swift darts-like motion, where the copepod reorients itself followed by resumed drifting (e.g. Tiselius and Jonsson, 1990). This "run-tumble" behavior was mathematically described by following a non-linear diffusive function by Visser and Kiørboe (2006). At a limited time scale, the drifting motility of the copepod seems linear – and the distance moved over time will increase linearly. This behavior is termed ballistic swimming (Visser and Kiørboe, 2006). Over time, as the copepod reorients itself (tumbles), the swimming path appears increasing more convoluted as the copepod change swimming direction.

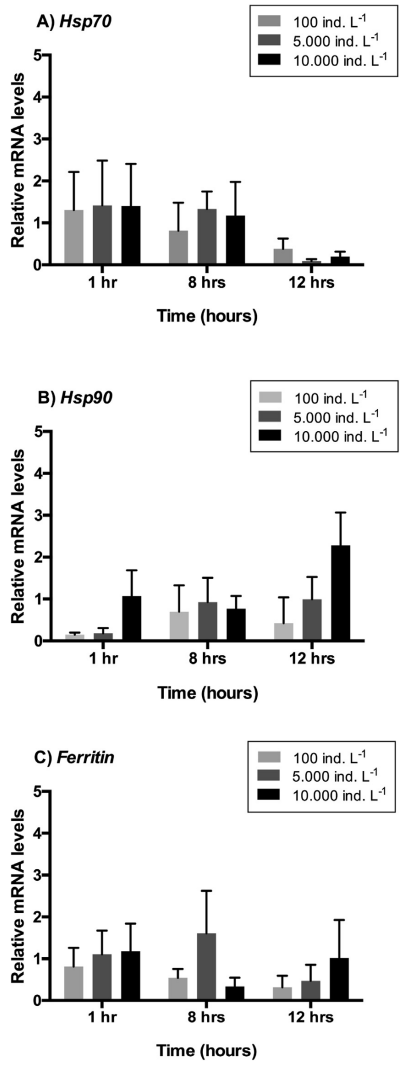

Fig. 4. Real-time quantitative PCR analysis of *hsp70*, *hsp90* and *ferritin* of the calanoid copepod *Acartia tonsa*. Densities of 100 (control, light grey), 5000 (grey) and 10,000 (black) ind. L^{-1} were used to examine gene expression levels of *ferritin* (A), *hsp70* (B) and *hsp90* (C). For each of the three densities, 4 biological replicates were collected after 1, 8 and 12 h of incubation (3 densities, 3 incubation-periods, 4 replicates = 36 samples in total). Each replicate consisted of 25 copepods. For real-time quantitative PCR, each biological replicate was performed with 3 technical replicates. Expression levels were normalized by *β- actin*. Error bars represent standard deviation (N = 4). None of the genes exhibited statistically significant changes in relative mRNA levels (Holm-Šidák, p > 0.05).

This swimming is termed diffusive (Berg and Brown, 1972). The swimming behavior of copepods exposed to various types of environmental stress is not well studied, and there are to our knowledge no reports in the literature on how the balance between ballistic and diffuse swimming is affected by stress.

We examined whether or not adults of *A. tonsa* exhibited changes in swimming behavior up to 10,000 ind. L^{-1}. We expected that the copepods at higher densities would bump into each other and thereby generate more convoluted swimming paths with increased diffusion rate and tumbling frequency. We did, however, not find the expected changes in swimming behavior except for the significantly different 100 ind. L^{-1} treatment. Here, all the copepods behaved within literature values from experiments conducted at much lower copepod density in terms of diffusion rates D (0.038 versus 0.036) and tumbling frequencies τ (1.78 versus 1.7) (Visser and Kiørboe, 2006).

Respiration rate is an integrative indicator of the overall metabolic activity, including motion, feeding and basal requirements (Kiørboe et al., 1985). Organisms have the ability to respond to stress, by increasing their energy demand, which can be reflected by an increased respiration rate. Since the high-densities we are using are much higher than the previously described *A. tonsa* densities (up to 6000 ind. L^{-1}), we expected several fold higher respiration rates in comparison to the low-density conditions used in the present study (e.g. Drillet et al., 2014b; Franco et al., 2017). However, biomass-specific respiration rates did not vary significantly with copepod density (Fig. 3).

The available studies regarding respiration rates in *A. tonsa* are primarily focusing on simultaneous feeding, swimming, fecundity and egg production aspects (e.g. Kiørboe, 2008; Kiørboe et al., 1985). Studies exploring the effect of stress on respiration rates of copepods are limited. Thor (2003) found that the respiration rate of *A. tonsa* (females) were reduced ~ 60% during starvation stress (8.5 nmol O_2 μmol C^{-1} h^{-1},17 °C) compared to non-starved copepods (of 20.0 nmol O_2 μmol C^{-1} h^{-1}, 17 °C). When food was reintroduced, the respiration rate increased within 8 h by ~40% compared to non-starved animals (32 nmol O_2 μmol C^{-1} h^{-1}, 17 °C) (Thor, 2003). The marine isopod, *Idotea balthica,* responded to an abrupt salinity decrease (30–10 psu) with an elevated respiration rate (~40% increase) within the first four hours after exposure. The elevated rate was sustained for 20 h, where after it decreased to the same level as prior the exposure after 30 h (Bulnheim, 1974). Since different stressors were shown to result in changes in respiration for *A. tonsa* and other crustaceans, we expected that the same would apply for density-related stress. Bulnheim (1974), and others, have shown that changes in respiration by crustaceans will occur within minutes to a few hours after stress exposure (e.g. Schapker et al., 2002; Tedengren et al., 1988). This suggests that the 12 h of incubation applied in the present study is considered sufficient to monitor changes in respiration.

Behavior and respiration are usually interrelated. If the behavior were changing toward more movements caused by high density, this would result in increased activity level and thereby be reflected in elevated respiration. Since there were no significant changes in either of the two physiological end-points, analysis of gene expression is a parameter that could give a more direct determination on whether the animals experience stress or not. The expression of stress related genes would change before the stress manifests as end-points like survival, fecundity and growth rate.

Dynamic transcription of genes allows rapid adaptation to external, environmental or physiological changes that affect the organisms. Gene expression analysis of selected genes can be used to monitor the physiological state under stressful conditions and give an early detection of when a stressor is affecting the organism in a negative manner. Several studies, using transcriptional biomarkers for stress, are available for copepods (reviewed in Lauritano et al., 2012). Genes encoding for chaperone molecules are usually up-regulated during stress conditions in order to preserve macromolecules in cells, and the gene expression patterns of chaperone genes could provide valuable information on the underlying mechanisms of copepod stress (reviewed in Lauritano et al., 2012). The genes for heat shock proteins, *hsp70* and *hsp90*, are widely up-regulated in response to a wide range of stressors and represent commonly used transcriptional stress biomarkers in copepods (e.g. Petkeviciute et al., 2015 and references herein). Ferritin is an iron-storage protein that protects macromolecules from damage by reactive oxygen species (Arosio and Levi, 2002; Hintze and Theil, 2006). Up-regulation of *ferritin* has been observed in response to nickel exposure, xenobiotics, and the resting states quiescence and diapause in copepods (Hansen et al., 2010a; Jiang et al., 2013; Nilsson et al., 2013; Tarrant et al., 2008). If adult individuals of *A. tonsa* experienced high-density conditions as being stressful, we expected a change in gene expression for the three chosen transcriptional biomarkers.

Comparison of the relative gene expression levels of *ferritin*, *hsp70* and *hsp90* between densities of 100 ind. L^{-1} did not vary over time (Holm-Šidák step down comparisons, $p > 0.05$). These densities represent low culture density and were therefore used as controls for statistical comparisons. None of the higher densities exhibited any significant changes in relative mRNA levels of *ferritin*, *hsp70* or *hsp90* for any of the incubation times ($p > 0.05$, Fig. 4). Compared to the positive stress responses for *A. tonsa* described in Petkeviciute et al. (2015) and Rahlff et al. (2017), with a fold increase of 63.8 and 185 in *hsp70* at heat shocks up to 30 °C, the observed fold changes in the present study (ranging from 0.028 to 3.074) cannot be considered a true stress response. Changes in gene expression of the heat shock proteins (*hsp70* and *hsp90*) as well as *ferritin* have been reported to happen within an hour to a few hours after exposure to different stressors (e.g. Duan et al., 2016; Petkeviciute et al., 2015). The incubation of up to 12 h is therefore sufficient to see a response on gene expression level.

Treatments at densities of 10,000 ind. L^{-1} during up to 12 h seems not to have a negative effect on adults of *A. tonsa* or induce a stress response that can be detected by behavior, respiration and gene expression. Long-term (>12 h) incubations at densities up to 6000 ind. L^{-1} have been shown to affect egg production and growth (Drillet et al., 2014b; Franco et al., 2017; Jepsen et al., 2007; Medina and Barata, 2004). The difficulties in rearing *A. tonsa* at high-densities might be caused by longer-term factors like oxygen and food depletion as well as accumulation of metabolic products (Jepsen et al., 2015; Ozaki et al., 2010; Støttrup and Norsker, 1997). High densities have, furthermore, been reported to increase egg cannibalism (Ban and Minoda 1994; Camus and Zeng 2009; Drillet et al., 2014a,b). However, *A. tonsa* egg cannibalism has been shown recently to be of minor importance when at high densities. In fact, just a few eggs ingested per copepod every 24 h was demonstrated when excess micro-algal food was present (personal communication, Minh Vu Thi Thuy, Roskilde University).

Other studies concerning the high-density effect on *A. tonsa* have focus on end-points like mortality, growth-rate, and egg production (Drillet et al., 2014b; Franco et al., 2017; Jepsen et al., 2007; Medina and Barata, 2004). The present study was planned to focus on somewhat alternative end-points (behavior, respiration, gene-expression) to act as a relatively fast and comprehensive set of parameters to discover the underlying causes for why *A. tonsa* is difficult to rear in high densities.

5. Conclusion

We found that adult individuals of the calanoid copepod, *A. tonsa*, after >30 years in culture do not exhibit signs on any stress

response at densities of up to 10,000 ind. L^{-1} when assessing swimming behavior, respiration rate, gene expression or mortality as physiological end points within a 12 h time frame. Limitations in copepod densities must be due to other parameters than acute stress.

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MANUSCRIPT II: Environmental stress responses of marine copepods: Evidence of experimental handling artifacts for *Acartia tonsa* (Dana)

Experimental handling of copepods can affect the gene expression of transcriptional biomarkers. This is problematic in relation to data analysis and interpretation, especially in studies of stress responses where handling can serve as a potential artefactual stressor. In this paper, we evaluated the whole-transcriptome response of *A. tonsa* to handling in comparison to a negative (no-handling) control, and a positive control (salinity shock), with evaluation both15 min and 24 hrs after exposure.

The paper demonstrates that handling does affect whole-transcriptome patterns of gene expression in *Acartia tonsa*. In addition, time after the exposure is a critical factor in designing experiments to measure stress responses at the transcriptional level. Some commonly used biomarkers were shown to exhibit low levels of differential expression for both handling and salinity shock 24 hrs after exposure, suggesting that these transcriptional responses may peak somewhere between 15 min and 24 hrs.

This work will raise awareness of the need for careful design of experimental procedures for transcriptional studies for copepods, and potentially other organisms. Especially, there should be raised precaution when using field-caught net-sampled individuals for gene expression studies, since some commonly-used biomarkers are affected by handling.

Environmental stress responses of marine copepods: Evidence of experimental handling artifacts for *Acartia tonsa* (Dana)

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Key words: Stress; Handling; Salinity; *Acartia tonsa*

ABSTRACT

Handling animals during experiments potentially affects the differential expression of genes chosen as biomarkers of sub-lethal stress. RNA sequencing was used to examine whole-transcriptome responses caused by laboratory handling of the calanoid copepod, *Acartia tonsa*. Salinity shock (S=35 to S=5) was used as positive stress control; individuals not exposed to handling or other stressors served as negative stress control. All copepods were grown from eggs to adults without being handled or exposed to any stressors prior the experiment. Survival of nauplii and adults was estimated or up to 10 min of exposure to handling stress and salinity shock. Only adults exhibited decreased survival (44±7% with 10 min of exposure) in response to handling stress and were selected for definitive experiments for RNA sequencing. After 10 min of experimental exposures to handling stress and salinity shock, adults were incubated for 15 min or 24 h at regular stock culture conditions. A small number of significantly differentially expressed genes (DEGs) were observed 15 min after exposure to handling stress (2 DEGs) and salinity shock (7 DEGs). However, 24 h after exposure, handling stress resulted in 276 DEGs and salinity shock resulted in 573 DEGs, of which 174 DEGs were overlapping between the treatments. Among the DEGs observed 24 h after exposure to handling stress and salinity shock, some commonly-used stress biomarkers appeared at low levels. This suggests that a stress-response was induced at the transcriptional level for these genes between 15 min and 24 h following exposure. Since handling stress clearly affects transcriptional patterns, it is important to consider handling when designing experiments, by either including additional controls or avoiding focus on impacted genes.

INTRODUCTION

Copepods provide a principal link of energy transfer from phytoplankton to higher trophic levels in the marine food web, and are preferred prey for predators, such as juvenile fishes and shrimps (Turner, 2004). Given the high natural abundance of copepods and their ubiquitous importance for marine ecosystems, understanding how stressors are affecting copepods is a central concern for estuarine and marine ecology. Copepods are widely used in environmental monitoring as indicators of ecosystem health (Beaugrand, 2009).

Transcriptional biomarkers that are commonly used to indicate sub-lethal effects of stress include enzymes detoxification enzymes (i.e. *cytochrome P450* and *Glutathionine-S-transferase*), as well as stress-related proteins, or chaperones, that protect macromolecules from damage (Amiard-Triquet and Berthet, 2015; Davies and Vethaak, 2012). In general, it is an overlooked issue that some of these biomarkers may respond to stress associated with handling, capture, collection, and other events associated with the experimental setup of both laboratory and field studies. Failure to consider the effects of handling in gene expression analysis from studies of environmental stress could cause erroneous assumptions about the data, by either increasing the risk of false positive results or by masking treatment-specific effects. Experimentally-induced and handling-related stress has been extensively studied in larger crustaceans (Fotedar and Evans, 2011). To our knowledge, only a few studies, e.g., Aruda et al. (2011) and Rahlff et al. (2017), have examined handling stress in copepods with targeted methods, which entail the evaluation of specific transcriptional biomarkers selected to evaluate certain stressors, typically by real-time quantitative PCR, as opposed to non-targeted methods, including transcriptome-wide analysis of gene expression.

The aim of this study is to examine the transcriptome-wide effects of handling stress on the calanoid copepod, *Acartia tonsa*. Because of the growing interest in *A. tonsa* as an indicator species for environmental monitoring, as well as use of the species as live-feed in aquaculture industries, there are important issues for selection of appropriate biomarkers and establishing an accurate baseline description of a non-stressed copepod (Kwok et al., 2015).

In both laboratory and field studies, plankton nets are commonly used for collection and size separation of copepod life stages (Rahlff et al., 2017; Uye and Kuwata, 1983). Because of this, the use of plankton nets was used to represent handling stress in this study.

A. tonsa is a robust species that, when acclimated, can persist in salinities ranging from 1 to 72 S, with an optimal salinity around 15 to 22 S (Cervetto et al., 1999). Even though *A. tonsa* is more tolerant of salinity variation compared to other *Acartia* species, abrupt change in salinity has been documented as a significant stressor (Calliari et al., 2006; Chinnery and Williams, 2004; Lance, 1964). Abrupt changes in salinity that exceed 10-15 S relative to the ambient level of *A. tonsa* have been shown to decrease survival more than 50% (Cervetto et al., 1999). For a positive-stress

control, we used an extreme salinity shock from S=35 to S=5 to provoke a response at both the transcriptional and physiological levels.

In addition to the overall lack of data in relation to handling stress, there is no information regarding to what extent handling will cause changes at the transcriptional level. Since this is a first approach of examining transcriptome-wide handling stress, the treatments used are "extreme", on order to ensure a response at the transcriptional level. The intention of the present study is to establish a foundation for future studies, in which handling stress can be described in greater detail.

MATERIALS AND METHODS

Stock culture

The strain of *A. tonsa* (identity code: DFH.AT1, Støttrup et al., 1986) has been in culture since 1981 when it was collected from Øresund (N 56°; E 12°; Denmark). The strain has been cultivated at Roskilde University (Denmark) in 60 L polyethylene tanks for >20 years under stable conditions (0.2 µm filtered seawater, S=35, 17°C, oxygen > 60%, dim lighting). The diet consists of the microalgae, *Rhodomonas salina* (identity code: K-1487), which is fed to *A. tonsa* on a daily basis in excess (>800 µg C L^{-1}; Berggreen et al., 1988). *R. salina* is cultivated in 2 L round-bottom flasks under stable temperature (17°C), with constant CO_2 supply and light (PAR ~80µE m^{-2} s^{-1}). The algal culture is diluted daily with Guillard's F/2 enrichment solution (Guillard and Ryther, 1962).

Experimental cultures

The experimental design comprised three treatments: control (no stress) (Fig. 1A), handling stress (Fig. 1B) and salinity shock (Fig. 1C).

Embryos of mixed age were harvested from three stock cultures and transferred to 2 L Nalgene© polycarbonate bottles (Thermo Fisher Scientific, USA) containing 0.2 µm filtered seawater (S=35), where they were incubated at 17°C for hatching. For each of the treatments triplicate cultivation flasks were set up as biological replicates. During cultivation, as well as during all experimental treatments, the flasks were kept at 17±1°C, with dim light and gentle aeration. *R. salina* were fed to the copepods *ad libitum* (>900 µg C L^{-1}; Berggreen et al., 1988) daily. Oxygen content was measured daily with a hand-held oxygen-probe (Handy Polaris 2, OxyGuard International A/S, Denmark) and exhibited values ranged from 6.9 - 7.5 mg O_2 L^{-1}.

Nauplii (body length: 130±16 µm, 4 days of development n=45) and adults (prosome length: 730±54 µm, 15 days of development) were used for determination of survival. Adults (764±42 µm, 15 days of development n=48) used for RNA sequencing were grown from eggs to the desired life stage without being handled. Prosome lengths were measured by photographing the copepods under a microscope (20 × magnification) and subsequently analyzing the images using the software package ImageJ (Schindelin et al., 2015).

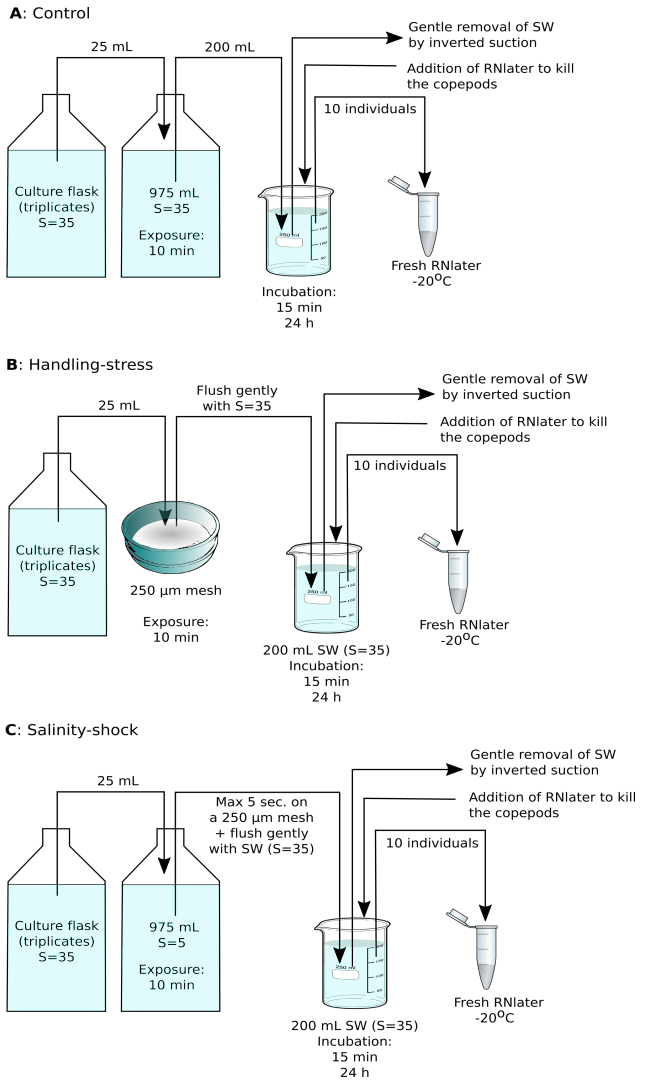

Figure 1: *Experimental setup for RNA sequencing. The control consisted of Acartia tonsa grown from eggs to adults prior to the experiment without being handled, followed by incubation at regular culture conditions for 15 min or 24 h, prior to fixation with RNAlater. The salinity shock treatment consisted of animals exposed to salinity S=5) for 10 min followed by incubation at stock culture conditions for 15 min or 24 h. In the handling stress treatment, adult individuals were placed on Nitex plankton net material with mesh size of 250 µm, and then kept at stock culture conditions for 15 min or 24 h before fixation with RNAlater. Each treatment was performed in triplicate, with each sample containing 10 adult individuals of A. tonsa.*

Experimental design

The experimental design was repeated twice, once to estimate survival and a second time for RNA sequencing (Fig. 1). The initial determination of survival was used to identify both the life-stage and the exposure time for the definitive experiment for RNA sequencing.

For the control, individuals (either nauplii or adults) were removed from the triplicate cultures in Nalgene© bottles using a 25 mL automatic pipettor (NS 29.2/32,Witeg, Germany) and transferred to 1 L glass beakers containing 975 mL seawater, with the same conditions as described for the stock culture. The copepods were held in the control treatment for 25 sec and 10 min before being transferred to new glass beakers. The copepods exposed to salinity shock were transferred to seawater with a salinity of S=5 and copepods exposed to handling stress were transferred to a Nitex plankton net (mesh size 54 µm for nauplii, 250 µm for adults) that was not submerged in water. For salinity shock and handling stress, the copepods were exposed for 25 sec, 1, 5 or 10 min before being transferred to glass beakers containing seawater with the same conditions as the stock culture. To distinguish between alive and dead, neutral red were added (15 mg/L seawater) for staining and the copepods were incubated for 15 min as described by Elliott & Tang (2009). After rinsing with distilled H_2O, dead and alive copepods were counted and survival was estimated. All three treatments were performed with 4 replicates.

Based on the results of the initial survival experiment, the definitive experiment for RNA sequencing was designed to include adult copepods and an exposure time of 10 min for handling stress or salinity shock, after which copepods were incubated for 15 min or 24 h at stock culture conditions. Seawater was then gently removed by inverted suction and the copepods were preserved in 20 mL RNAlater. Ten individuals from each triplicate experimental treatment were immediately transferred to 1 mL fresh RNAlater and stored at -20°C (Figure 1A-C).

Additionally, survival was estimated for copepods 24 h after exposure to 10 min salinity shock nd handling stress with a control of non-handled individuals, as described for the initial survival estimation.

Statistical analysis and preparation of graphics were done using R (www.R-project.org, version 3.4.0). From the counts of dead copepods, survival (%) was calculated for each exposure time and analyzed by linear regression. Differences between treatments were analyzed based on two-tailed t-tests of the regression coefficients (Sokal and Rohlf, 1995).

RNA extraction, library preparation and sequencing

Total RNA was extracted from *A. tonsa* using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). For each treatment (control, handling stress, salinity shock), three biological replicates were analyzed, each consisting of 10 pooled individuals (prosome length = 758±67 µm).

After removal of excess RNAlater (Sigma Aldrich, St. Louis, MO, USA), the copepods were homogenized in 50µL RLT buffer using disposable micro-pestles, after which 550 µL RLT buffer was added. The samples were vortexed for 1-2 sec,

and then processed according to the manufacturer's protocol, with a final elution volume of 30 µL in RNase-free water.

RNA quality was assessed using an Agilent Tapestation 2200 with RNA High Sensitivity Assay (Agilent Technologies, Santa Clara, CA, USA). DNase treatment was not done, since previous extractions treated with the Turbo DNA-free kit (Ambion, Life Technologies, Carlsbad, CA, USA) exhibited significant degradation. The RNA quality profiling of *A. tonsa* exhibited a merged peak of 18S rRNA and 28S rRNA, presumably resulting from 'hidden break' in 28S rRNA typical of many arthropods, which causes 28S rRNA to run at about the same size as 18S rRNA (McCarthy et al., 2015).

Library preparation was done using 360 ng total RNA from each sample following the manufacturer's protocol for the Illumina Stranded mRNA Library Preparation Kit (Illumina, Inc., San Diego, CA, USA). The libraries constructed from the 18 samples (3 treatments x 3 replicates x 2 incubation times) were multiplexed and sequenced in 2 runs across 4 lanes on the NextSeq500 platform (Illumina, San Diego, CA, USA), with a mid-output 150 cycle kit (FC-404-2001, Illumina, Inc., San Diego, CA, USA) with 75 bp paired-end reads and a sequencing depth of 25 million reads per sample. Library preparation and sequencing were carried out at the Center for Genome Innovation at the University of Connecticut (Storrs, CT, USA).

A reference transcriptome was determined from RNA extracted from a single individual of *A. tonsa* (female, prosome length 722 µm) selected at random from the control with 24 h of incubation. Total RNA (140 ng) was sequenced in 4 lanes on a NextSeq500 platform using a mid-output 300 cycles kit (FC-404-2003, Illumina, Inc., San Diego, CA, USA), with 150 bp paired-end reads resulting in ~350 million reads.

Table 1. *Assembly, validation, annotation and pseudo-alignment statistics*

Assembly					
#Raw reads	354,098,566				
#Reads after QC	225,217,658				
#Trinity contigs	60,662				
#Trinity components	27,171				
N50	1,874 bp				
Median contig length	790 bp				
Average contig length	1,222 bp				
Validation					
E90N50	2,731 bp				
E69N50	3,075 bp				
Bowtie2 realign.	90.35%				
BUSCO	*Complete*	*Single-copy*	*Duplicate*	*Fragmented*	*Missing*
	99.0%	44.9%	54.1%	0.7%	0.3%
Annotation					
Trinotate	45%				
Blast2Go	163 non-Trinotate-annotated differential expressed transcripts				
Pseudo-alignment					
Kallisto	82.2±2.6 %				

205 ***De novo* transcriptome assembly and differential gene expression analysis**
206 FastQC (ver. 0.7, Andrews, 2010) was used to validate the quality of the raw
207 sequence reads. Illumina adapter sequences and low-quality reads (Phred score < 20)
208 were removed using Trimmomatic (ver. 3; Bolger et al., 2014) in paired-end mode,
209 with a sliding window across an average of 4 bases. Initial read biases, introduced by
210 random hexamer priming under cDNA synthesis, were corrected by removing the first
211 12 bp of each read (Hansen et al., 2010). Reads > 50 bp after quality trimming were
212 retained, resulting in a total of ~225 million reads. The reference transcriptome was
213 assembled *de novo* with Trinity (ver. 2.3.2; Grabherr et al., 2011) using default
214 parameters for paired-end reads, with normalization to decrease run time and memory
215 requirements.
216 The completeness of the reference transcriptome was evaluated using the
217 Benchmarking Universal Single-Copy Orthologs (BUSCO, ver. 2; Simão et al. 2015),
218 which defines a set of eukaryotic core genes to test the proportion and completeness
219 of these genes in the transcriptome assembly. Bowtie2 (ver. 2.2.6; Langmead and
220 Salzberg, 2012) was used to examine the RNA sequencing read representation of the
221 assembly by realigning the input reads to the *de novo* transcriptome. Contig N50 and
222 E90N50 statistics were computed based on the scripts included in the Trinity software
223 package, as well as transcript abundance estimation using Kallisto (ver. 0.43.0; Bray
224 et al. 2016). The *A. tonsa* Transcriptome Shotgun Assembly project has been
225 deposited at DDBJ/EMBL/GenBank under the accession GFWY00000000. The
226 version described in this paper is the first version, GFWY01000000.
227 The Trinotate annotation pipeline (ver. 3.0.2; Haas et al., 2013) was used to annotate
228 the reference transcriptome using Swissprot (Bairoch and Apweiler, 1999); Pfam
229 (Finn et al., 2010); eggNOG (Powell et al., 2012); KEGG (Kanehisa et al. 2012); and
230 Gene Ontology (Ashburner et al., 2000).
231
232 The reads from the experimental samples were pseudoaligned (i.e., rapid
233 determination of compatibility between reads and targets, without the need for
234 alignment) to the reference transcriptome and quantified using Kallisto (ver. 0.43;
235 Bray et al., 2016), with 100 rounds of bootstrapping. The bootstrapping option in
236 Kallisto accounts for technical variability and is used to estimate the probability of
237 correct assignment to a transcript. Differential gene (and transcript) analysis was
238 performed with Sleuth (ver. 0.29; Pimentel et al., 2017), using the likelihood ratio test
239 (LRT) and the Wald test in R to estimate significant results (ver. 3.4.0; Anon, 2017).
240 Statistically significant (q-values < 0.05) differential gene expression is reported as
241 beta values, which are bias estimators of the fold-change that accounts for the
242 technical variability of transcripts and are reported as natural log values (Pimentel et
243 al. 2017) (See S1b for results of Sleuth analysis of non-annotated transcripts.)
244 The Trinity transcript identifications for non-annotated transcripts were added into the
245 gene-level analysis in Sleuth. Transcripts showing statistically significant differential
246 expression (q-value < 0.05) were isolated and annotation was attempted using
247 Blast2Go and the RefSeq database with the arthropod taxonomy filter, in order to
248 maximize the proportion of identified genes in the analysis (Gotz et al., 2008). Based

on the BlastX results ($E \leq 10^{-3}$), gene symbols for identified transcripts were added to the gene-level analysis in Sleuth. Transcripts that could not be identified were excluded from the analysis of differential gene expression.

Functional enrichment of differentially expressed genes was performed using clusterProfiler (ver. 3.6.0, Yu et al., 2012) for gene ontologies (GO) of cell compartments (CC), biological processes (BP) and, molecular functions (MF). The genes annotated by Trinotate from the reference transcriptome were used as background gene-list. Differentially up- and down-regulated genes for handling stress and salinity shock (15 min and 24 h after exposure) were used as input data. The functional enrichment was performed with a Fisher Exact test (p-value < 0.05, FDR < 0.1). Graphs and subsequent data handling was done in R using the ggplot2 package (ver. 2.2.1).

RESULTS

Survival

Survival of *A. tonsa* nauplii was significantly ($p < 0.001$) affected by salinity shock (i.e., exposure to S=5 for up to 10 min), in comparison to the control (Fig. 2E, Table 2E). Naupliar survival in the control was 91±4%, while survival after salinity shock after 25 sec exposure was 95±1%, declining to 22±4% after 10 min exposure. Naupliar survival after handling stress was 97±2% for exposure up to 10 min, which did not differ from the control, with an average survival of 97±1% (Fig. 2D, Table 2D). Survival for handling stress and salinity shock differed significantly by regression analysis ($p < 0.001$) (Fig. 2F, Table1F).

Adult individuals of *A. tonsa* were highly significantly affected both by salinity shock ($p < 0.001$) and handling stress ($p < 0.001$) for exposure up to 10 min (Fig. 2A, B, Table 2A, B) in comparison to the control, which exhibited survival of 98±1% up to 10 min exposure time. Survival declined from 91±2% after 25 sec to 56±7% after 10 min of exposure to handling stress. Survival declined in the salinity shock from 92±1% after 25 sec to 70±5% after 10 min of exposure. Survival was significantly higher from salinity shock than handling stress estimated by regression analysis ($p < 0.05$, Fig. 2C, Table 2C).

Survival of salinity shock and handling stress was estimated 24 h post 10 min of exposure. The control exhibited a survival of 99±1.4%, salinity shock 47±1.6% and handling stress 54±1.7%. In the samples with 34±1.2% exhibited physical damage of the antenna, setae and antennules. Of the damaged individuals, 74±2.7% were categorized as dead during staining. For the salinity shock samples, only 4±3.2% exhibited damage, of which 53 ±1.5% was categorized as dead.

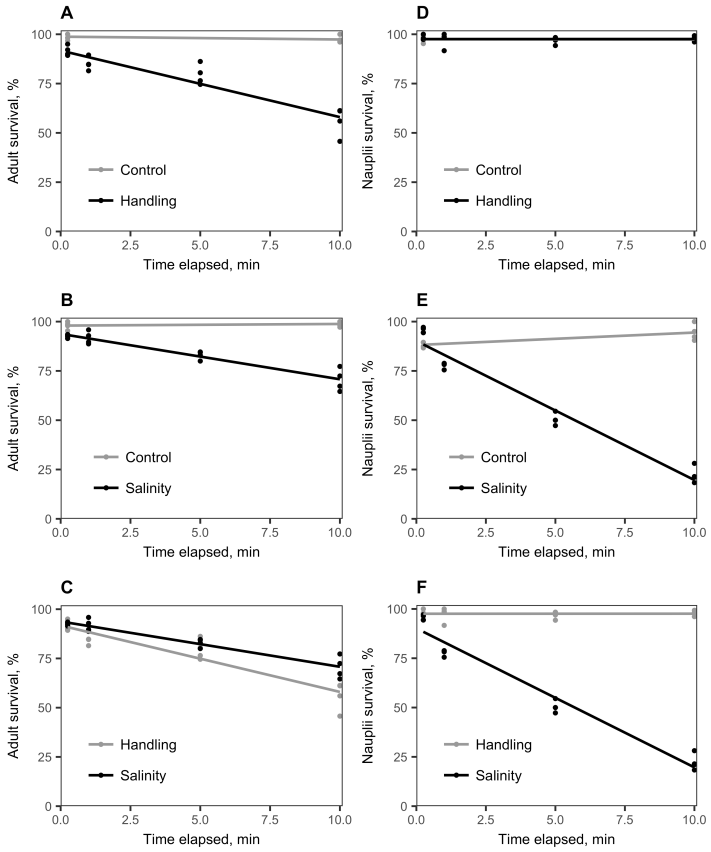

Figure 2: *Linear regression analysis of survival (%) versus time elapsed for various treatments compared to the control treatment. The handling stress treatment consisted of placing copepods on a Nitex plankton net (adult mesh size: 250 µm; nauplii mesh size: 54 µm). The salinity shock treatment consisted of exposure of copepods to S=5.*

A) Adult individuals of Acartia tonsa exposed to handling stress (black) versus control (grey). The two treatments differed statistically significantly from each other (Table 2, A).

B) Adult individuals of A. tonsa exposed to a salinity shock (black) versus the control (grey). Survival in the two treatments differed statistically significantly (Table 2, B).

C) Adult individuals of A. tonsa exposed to salinity shock (black) versus individuals exposed to handling stress (grey). Survival in the two treatments differed statistically significantly from each other (Table 2, C).

D) A. tonsa nauplii exposed to handling stress (black) versus control (grey). Survival in the two treatments did not differ statistically significantly from each other (see table 2, D).

E) A. tonsa nauplii exposed the salinity shock (black), versus control (grey). The two treatments differed statistically significantly from each other (Table 2, E).

F) A. tonsa nauplii exposed to the salinity shock (black) versus individuals exposed to handling stress (grey). The two treatments differed statistically significantly from each other (Table 2, C).

Table 2. *T-test comparison of regression coefficients for linear regressions shown in Fig. 2. A) Adults, negative control versus handling stress treatment; B) Adults, control versus salinity shock treatment; C) Adults, handling stress versus salinity shock; D) Nauplii, control versus handling stress treatment; E) Nauplii, control versus salinity shock; F) Nauplii, handling stress versus salinity shock. Slope corresponds to the regression coefficients of the linear regressions shown in Fig. 2.* SE_{slope} *is the standard error of the given regression coefficients. N is the sample size. SSE is the error sum of squares. T is the calculated "T-test" value and df is the degree of freedom where p is the probability value.*

Graph		Treatment	Equation	R^2	SE_{slope}	N	SSE (b1-b2)	T-test	df	p
Adults	*A*	*Control (handling stress)*	98.78 - 0.14x	0.18	0.123	8	0.380	8.5042	20	<0.001
		Handling stress	91.75 - 3.38x	0.86	0.359	16				
	B	*Control (salinity shock)*	97.97 - 0.08x	0.07	0.124	8	0.243	9.8029	20	<0.001
		Salinity shock	93.77 - 2.30x	0.90	0.209	16				
	C	*Handling stress*	91.75 - 3.375x	0.86	0.359	16	0.416	-2.5815	28	<0.05
		Salinity shock	93.77 - 2.30x	0.90	0.209	16				
Nauplii	*D*	*Control (handling stress)*	97.48 - 0.02x	0.01	0.099	8	0.173	-0.1197	20	Not significant different
		Handling stress	97.56 + 0.01x	2.8E-05	0.141	16				
	E	*Control (salinity shock)*	88.12 + 0.63x	0.57	0.225	8	0.452	17.0094	20	<0.001
		Salinity shock	90.17 - 7.05x	0.96	0.392	16				
	F	*Handling stress*	97.56 +0.003x	2.8E-05	0.141	16	0.417	16.9311	28	<0.001
		Salinity shock	90.18 - 7.05x	0.96	0.392	16				

306

307 ***De novo* reference transcriptome and annotation**

308 A total of ~225 million reads >50 bp in length (after quality trimming) was retained
309 for reference transcriptome assembly. The *de novo* assembled transcriptome consisted
310 of 60,688 contiguous consensus sequences (contigs) grouped into 27,171 Trinity
311 components ("genes") with a GC content of 38.49%. Statistics based on all transcript
312 contigs had an N50 value of 1,874 bp, with an average contig length of 1,222.45 bp
313 from for a total of 74,188,026 assembled bases (Table 1).
314 The quality of different Trinity transcriptome assemblies was evaluated using
315 Bowtie2 for realignment of the reads to the reference, BUSCO evaluation of
316 completeness, and E90N50 profiles of contig length (Table 1).
317 Of the reference input RNA sequencing reads realigned with Bowtie2, 90.35% were
318 represented in the assembly of the chosen reference transcriptome (Table 1). The
319 remaining unassembled reads, likely corresponded to low-expressed transcripts with
320 insufficient coverage to enable assembly, was of low quality or resulted from aberrant
321 reads.
322 Completeness of the transcriptome was examined by searching for single copy
323 orthologs with 99.0% BUSCO scores. Results from 303 BUSCO groups searched

were: single-copy: 44.9%; duplicated: 54.1%, fragmented: 0.7%; missing: 0.3% (Table 1).

The Ex90N50 transcript contig length of 2,731 bp was computed by combining Kallisto (ver. 0.43.0; Bray et al., 2016) and the ExN50 statistic script included in the Trinity package. Since N50 statistics discard read coverage, E90N50 gave an indication of whether deeper sequencing would result in higher quality assembly. The ExN50 profile peaked at N69, with a contig length of 3,075 bp (Table 1).

Considering the overall realignment, BUSCO profile, and ExN50 profile, we evaluated the reference transcriptome to be of acceptable quality for the differential gene expression analysis. The Trinotate annotation pipeline resulted in identification of 45% of the assembled Trinity transcripts (Table 1). The remaining 55% of unidentified transcripts were excluded from the differential gene expression analysis, after ensuring that the significantly differentially expressed transcripts could not be identified in any way. (See S1b for Sleuth analysis including unidentified transcripts.) The average pseudo-alignment of the experimental sample reads to the reference transcriptome using Kallisto was 82.2±2.6% (Table 1).

From the Trinotate annotation, 80,047 Gene Ontologies (GO) and 24,463 Kyoto Encyclopedia of Genes and Genomes (KEGG) ontology (KO) terms were associated with the genes. It should be noted that multiple GO-terms could be assigned to the same gene. The GO-terms consisted ($p < 0.05$, FDR < 0.2) of 48% Biological Processes (BP), 30% Cellular Compartments (CC) and 22% Molecular Functions (MF) (see S3a for all enriched GO terms).

Table 3: *Overview of the number (#) of differentially expressed genes (DEGs).*

# DEGs for following treatments:		**Handling**	**Salinity shock**
15 min	*Total*	2	7
	Up-regulated	1	6
	Down-regulated	1	1
	Treatment specific	2	7
24 h	*Total*	276	396
	Up-regulated	177	221
	Down-regulated	99	175
	Treatment specific	102	222
# Overlapping DEGs			
Handling stress vs. salinity shock, 15 min		0	
Handling stress vs. salinity shock, 24 h		174	
Handling stress, 15 min vs. 24 h		0	
Salinity shock, 15 min vs. 24 h		2	

Differentially expressed genes

The patterns of differential gene expression for copepods from the handling stress, salinity shock and control 15 min after exposure were not distinctive in the Principal Component Analysis (PCA, Fig. 4A). The control exhibited more distinct clustering from handling stress and salinity shock treatments 24 h after exposure (Fig. 4B). This suggests that the time following exposure has a significant effect on gene expression.

Two differentially expressed genes (DEGs; q-value < 0.05), one up- and one down-regulated, were identified 15 min after exposure to handling stress (Fig. 3 & 5, Table 3; see S1a for b-values). The up-regulated DEG (*IPPK*, *Inositol-pentakisphosphate 2-* had two enriched BPs: Melanosome transport and determination of left/right symmetry (Table 4), two CCs: Ciliary basal body and Centrosome (Table 4), one MF: Inositol pentakisphosphate 2-kinase activity (Table 4), and two KOs: Phosphatidylinositol signaling system and Inositol phosphate metabolism (Table 4). The down-regulated DEG (*SIDT1*, *SID1 transmembrane family member 1*) was enriched in dsRNA transport (BP, Table 4) and RNA trans-membrane transporter activity (MF, Table 4).

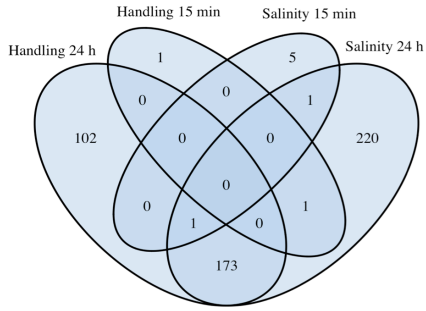

Figure 3: *Comparative analysis of differential expressed genes (DEGs) in Acartia tonsa exposed to 10 min handling stress and 10 min salinity shock after 15 min or 24 h. The Venn diagram was constructed from overlapping transcripts in R with the library VennDiagram (ver. 1.6.17).*

Handling stress resulted in 276 DEGs 24 h after exposure, of which 177 were up-

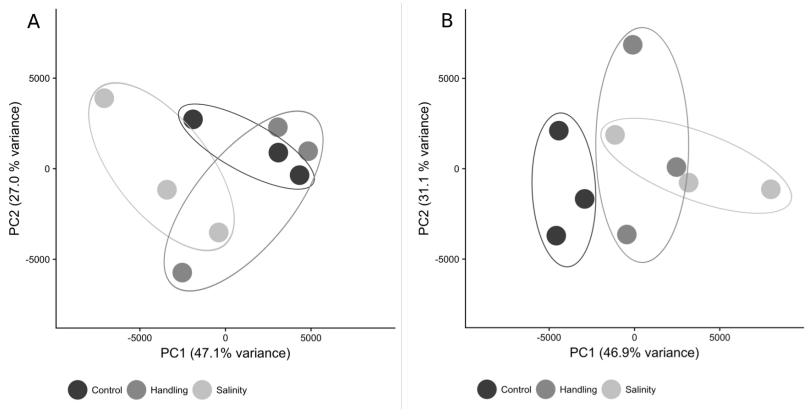

Figure 4. *Principal component analysis (PCA) dimensions 1 and 2 of expression values for all contigs in each sample. Symbols are: control (black), handling stress (dark grey); salinity shock (light grey). A) After 10 min; B) After 24 h. PCA was computed in R using the Sleuth package.*

regulated and 99 down-regulated (Fig. 3 & 5, Table 3). None of the same DEGs were overlapping 15 min and 24 h after exposure. The salinity shock resulted in 7 DEGs (6 up- and 1 down-regulated) 15 min after exposure, which increased to 396 DEGs (221 up- and 175 down-regulated) 24 h after exposure (Fig. 3, Table 3).

Among the up-regulated DEGs 15 min after exposure to salinity shock, the majority of enriched GO-terms were transport mechanisms, especially related to ER homeostasis and proteins (see S3a for the full list, Table 4 for top 10 of the GO-terms with most involved genes). The remainder of the enriched GO-terms were related to metabolic, homeostatic, and developmental processes (Table 4, S3a). The majority of these processes took place in the lysosomes (Table 4, S3a), Golgi apparatus (Table 4, S3a), and vesicles (Table 4, S3a).

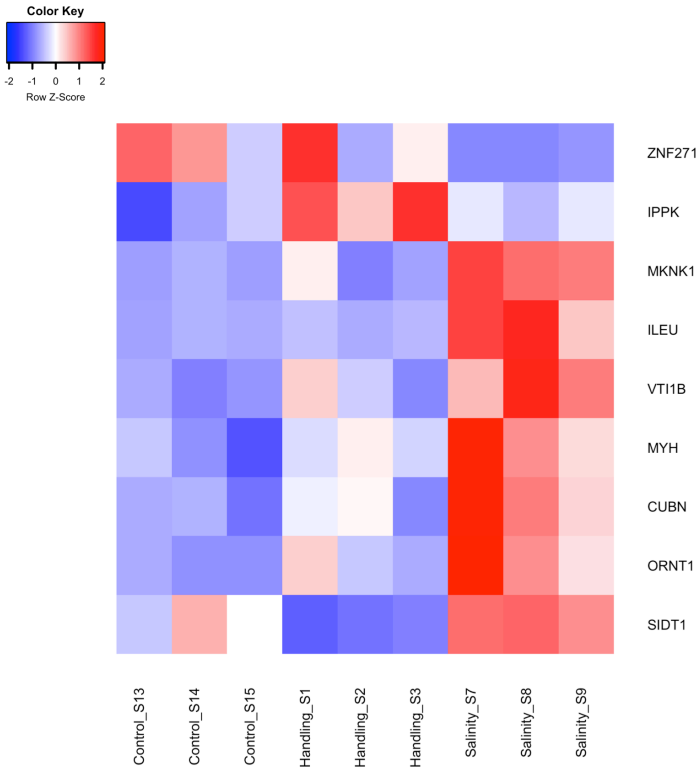

Figure 5: *Heat map of differential expressed genes (q<0.05) for the handling stress and salinity shock treatments relative to the control after 15 min. The color scale of transcript expressions is normalized as z-scores were up-regulated transcripts are red and down-regulated are blue. The heat map was generated in R using the library gplots (version 3.0.1).*

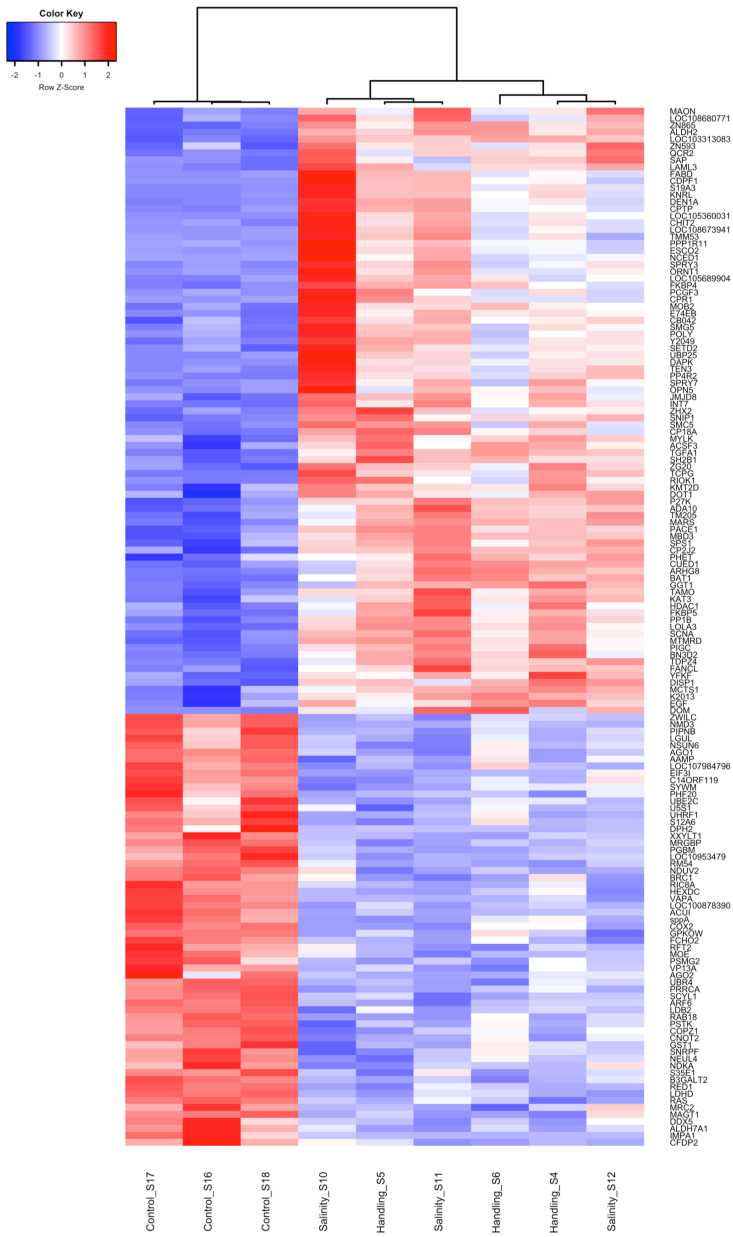

Figure 6: *Heat map of overlapping differential expressed genes ($q < 0.05$) for the handling stress and salinity shock treatments relative to the control after 24 h. Similarities between samples are given as a dendrogram for hierarchical clustering with Pearson correlation with complete distance determination. The color scale of transcript expressions is normalized as z- scores, where up-regulated transcripts are red and down-regulated are blue. The heat map was generated in R using the library gplots (version 3.0.1)*

388

The three enriched KO-terms were related to signaling pathways and vitamin digestion and absorption (Table 4).

Two up-regulated DEGs (*ORNT1*, *Ornithine Transporter 1*; *MYH*, *Myosin Heavy chain*; S1a, Table 5) were overlapping 15 min and 24 h after exposure to salinity shock. ORNT was enriched in mitochondrial ornithine transport (BP, Table 4) taking place in the mitochondrial inner membrane (CC, Table 4), and related to two enriched MFs: L-ornithine transmembrane transporter activity and thiol-dependent ubiquitinyl hydrolase activity (Table 4).

Table 4. *Enriched gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes ontologies (KEGG, KO) for differential expressed genes (DEGs) 15 min post exposure to handling-stress and salinity shock. The top 10 (if there is 10) GO-terms with most genes involved are here shown for up- and down regulated DEGs for each treatment. The DEGs (genes) sorted by number of involved genes, and then based on p-values with most significant enrichments (p). Cat.: Ontology category, can be BP: Biological Process, CC: Cellular Compartment, MF: Molecular Function, or KEGG. p is the false discovery rate corrected p-value (FDR < 0.2). Change if the change in gene expression relative to the control (up or down regulated). ID is the ontology ID. Genes gives the abbreviations for DEGs; full names can be found in S2a. None of the DEGs 15 min post exposure is overlapping between handling-stress and salinity shock. Enrichment of GO and KEGG terms were done using the R-package ClusterProfiler (ver. 3.6.0, Yu et al., 2012) with the Trinotate annotated transcriptome as background gene list.*

Description	Cat.	p	Change	ID	Genes
15 min post exposure to 10 min handling-stress (2 DEGs in total)					
Inositol pentakisphosphate 2-kinase activity	MF	0.0001	Up	GO:0035299	*IPPK*
Melanosome transport	BP	0.007	Up	GO:0032402	*IPPK*
Determination of left/right symmetry	BP	0.003	Up	GO:0007368	*IPPK*
Ciliary basal body	CC	0.004	Up	GO:0036064	*IPPK*
Centrosome	CC	0.02	Up	GO:0005813	*IPPK*
RNA transmembrane transporter activity	MF	0.0001	Down	GO:0051033	*SIDT1*
dsRNA transport	BP	0.001	Down	GO:0033227	*SIDT1*
Phosphatidylinositol signaling system	KEGG	0.005	Up	KO:04070	*IPPK*
Inositol phosphate metabolism	KEGG	0.006	Up	KO:00562	*IPPK*
15 min post exposure to 10 min salinity shock (7 DEGs in total)					
Lysosomal membrane	CC	0.002	Up	GO:0005765	*VTI1B/CUBN*
Golgi apparatus	CC	0.01	Up	GO:0005794	*VTI1B/CUBN*
L-ornithine transmembrane transporter activity	MF	0.0007	Up	GO:0000064	*ORNT1*
Mitochondrial ornithine transport	BP	0.0007	Up	GO:0000066	*ORNT1*
Cobalamin transporter activity	MF	0.0007	Up	GO:0015235	*CUBN*
Extrinsic component of external side of plasma membrane	CC	0.0007	Up	GO:0031232	*CUBN*
Cobalamin transport	BP	0.001	Up	GO:0015889	*CUBN*
Hemoglobin import	BP	0.001	Up	GO:0020028	*CUBN*
Hemoglobin binding	MF	0.001	Up	GO:0030492	*CUBN*
Endocytic vesicle membrane	CC	0.001		GO:0030666	*CUBN*
Vitamin digestion and absorption	KEGG	0.006	Up	KO:04977	*MKNK2*
HIF-1 signaling pathway	KEGG	0.02	Up	KO:04066	*MKNK1*
Insulin signaling pathway	KEGG	0.02	Up	KO:04910	*MKNK1*

Table 5: *Enriched gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes ontologies (KEGG, KO) for overlapping differential expressed genes (DEGs) 24 h post exposure to handling-stress and salinity shock, as well as overlapping DEGs 15 min and 24 h post exposure to salinity shock. The top 10 (if there is 10) GO-terms with most genes involved are here shown for up- and down regulated DEGs. The DEGs (genes) sorted by number of involved genes, and then based on p-values with most significant enrichments (p). Cat.: Ontology category, can be BP: Biological Process, CC: Cellular Compartment, MF: Molecular Function, or KEGG. p is the false discovery rate corrected p-value (FDR < 0.2). Change if the change in gene expression relative to the control (up or down regulated). ID is the ontology ID. Genes gives the abbreviations for DEGs; full names can be found in S2a. None of the DEGs 15 min post exposure is overlapping between handling-stress and salinity shock. Enrichment of GO and KEGG terms were done using the R-package ClusterProfiler (ver. 3.6.0, Yu et al., 2012) with the Trinotate annotated transcriptome as background gene list.*

Description	Cat.	p	Change	ID	Genes
Overlapping DEGs, 24 h post exposure to handling stress and salinity shock (174 DEGs in total)					
Neurogenesis	BP	0.02	Up	GO:0022008	*HDAC1/PP4R2/DOM/LOLA3/SPS1*
Cellular response to DNA damage stimulus	BP	0.02	Up	GO:0006974	*UBP25/INT7/FANCL/BD1L1/SMC5*
Chromosome	CC	0.01	Up	GO:0005694	*ZG20/INT7/SETD2/BD1L1*
Oogenesis	BP	0.02	Up	GO:0048477	*HDAC1/DOM/E74EB/BRN*
Ubiquitin protein ligase binding	MF	0.04	Up	GO:0031625	*SMG5/FANCL/TDPZ4/TS101*
Response to estradiol	BP	0.003	Up	GO:0032355	*MBD3/GGT1/ALDH2*
Chaperone-mediated protein folding	BP	0.003	Up	GO:0061077	*TCPG/FKBP4/FKBP5*
mRNA export from nucleus	BP	0.02	Up	GO:0006406	*SMG5/NU155/NUP62*
Chromatin	CC	0.02	Up	GO:0000785	*ESCO2/HDAC1/MBD3*
Transcription corepressor activity	MF	0.02	Up	GO:0003714	*ZHX2/HDAC1/TS101*
Plasma membrane	CC	0.006	Down	GO:0005886	*SYWM/LGUL/CNOT2/FCHO2/AAMP/S12A6/NDKA/ARF6/MAGT1/RIC8A/RAS/UBR4/PGBM*
Regulation of transcription from RNA polymerase II promoter	BP	0.0003	Down	GO:0006357	*MRGBP/BRC1/LGUL/LDB2/CNOT2/DDX5*
Positive regulation of transcription from RNA polymerase II promoter	BP	0.02	Down	GO:0045944	*UHRF1/BRC1/AGO2/PHF20/DDX5/AGO1*
GTP binding	MF	0.007	Down	GO:0005525	*RAB18/U5S1/NDKA/ARF6/RAS*
Double-stranded RNA binding	MF	0.0004	Down	GO:0003725	*RED1/AGO2/AGO1*
RNA secondary structure unwinding	BP	0.002	Down	GO:0010501	*AGO2/DDX5/AGO1*
Extracellular matrix	CC	0.003	Down	GO:0031012	*U5S1/DDX5/PGBM*
Angiogenesis	BP	0.004	Down	GO:0001525	*AAMP/S12A6/PGBM*
Intracellular ribonucleoprotein complex	CC	0.007	Down	GO:0030529	*AGO2/DDX5/AGO1*
Brain development	BP	0.008	Down	GO:0007420	*RED1/RAB18/PGBM*
Aminoacyl-tRNA biosynthesis	KEGG	0.006	Down	KO:00970	*SYWM/PSTK*

Pyruvate metabolism	KEGG	0.01	Down	KO:00620	*LGUL/LDHD*
Spliceosome	KEGG	0.02	Down	KO:03040	*U5S1/DDX5*
RNA transport	KEGG	0.03	Down	KO:03013	*NMD3/EIF3I*
Other glycan degradation	KEGG	0.03	Down	KO:00511	*HEXDC*
Proteoglycans in cancer	KEGG	0.04	Down	KO:05205	*PGBM/DDX5*
Vitamin digestion and absorption	KEGG	0.04	Down	KO:04977	*RFT2*
Overlapping DEGs, 15 min and 24 h post exposure to salinity shock (2 DEGs in total)					
Mitochondrial ornithine transport	BP	0.0002	Up	GO:0000066	*ORNT1*
Mitochondrial inner membrane	CC	0.03	Up	GO:0005743	*ORNT1*
L-ornithine transmembrane transporter activity	MF	0.0002	Up	GO:0000064	*ORNT1*
Thiol-dependent ubiquitinyl hydrolase activity	MF	0.002	Up	GO:0036459	*ORNT1*

The overlap between handling stress and salinity shock 24 h after exposure was 174 DEGs. For the overlapping 112 up-regulated DEGs, 223 GO-terms were enriched terms (see S3a for the full list, Table 5 for top 10 of the GO-terms with most involved genes). No KO-terms were enriched. For the 62 down-regulated DEGs, 176 GO- and 7 KEGG terms were enriched (S3a, Table 5).

The remaining non-overlapping 102 and 202 DEGs for handling stress and salinity shock 24 h post exposure, respectively, may be stressor-specific see S3a for the full list, Table 6 for top 10 of the GO-terms with most involved genes). For the 65 up-regulated non-overlapping DEGs 24 h post exposure to handling, 151 GO and 3 KO-terms were enriched. The 37 down-regulated DEGs had 94 enriched GO and 12 KO terms (s3a, Table 6). For non-overlapping DEGs 24 h post exposure to salinity shock, 109 were up- and 113 were down-regulated. The up-regulated DEGs resulted in the enrichment of 173 GO and 8 KO terms, while the down-regulated DEGs resulted in 226 GO and 2 KO terms (s3a, Table 6).

Inclusion of the Trinity transcript identifications in the Sleuth analysis (S1b) resulted in 350 DEGs (244 up- and 106 down-regulated) for the handling stress treatment after 24 h. The salinity shock resulted in 573 annotated DEGs (376 up- and 197 down-regulated) 24 h after exposure.

DISCUSSION

The results from our examination of transcriptome-wide responses and survival in relation to handling stress and salinity shock in *A. tonsa* clearly indicate that handling stress is a significant factor for experimental manipulation of this species. Failure to consider this parameter in, for instance, biomarker-related transcriptional studies is likely to lead to false interpretation of the stressors' impact.

The nauplii were not affected by handling stress in terms of survival. This may be because of their small size and lack of fragile appendages, allowing a boundary layer of seawater to form around them as protection from physical interaction with the plankton net. Since handling stress did not negatively impact nauplii survival, we used only adult individuals of *A. tonsa* for the definitive experiments for RNA sequencing analysis.

Table 6: *Enriched gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes ontologies (KEGG, KO) for non-overlapping differential expressed genes (DEGs) 24 h post exposure to handling-stress and salinity shock. The top 10 (if there is 10) GO-terms with most genes involved are here shown for up- and down regulated DEGs for each treatment. The DEGs (genes) sorted by number of involved genes, and then based on p-values with most significant enrichments (p). Cat.: Ontology category, can be BP: Biological Process, CC: Cellular Compartment, MF: Molecular Function, or KEGG. p is the false discovery rate corrected p-value (FDR < 0.2). Change if the change in gene expression relative to the control (up or down regulated). ID is the ontology ID. Genes gives the abbreviations for DEGs; full names can be found in S2a. None of the DEGs 15 min post exposure is overlapping between handling-stress and salinity shock. Enrichment of GO and KEGG terms were done using the R-package ClusterProfiler (ver. 3.6.0, Yu et al., 2012) with the Trinotate annotated transcriptome as background gene list.*

Description	Cat.	p	Change	ID	Genes
24 h post exposure to handling-stress (102 non-overlapping DEGs in total)					
ATP binding	MF	0.002	Up	GO:0005524	*PTPA/CDK8/ULK3/TRIO/PRS6B/SKIV2/CDK12/MRP2/PRP16/CDC48/MOS/CNNM2/PRPS1/MAST1*
RNA polymerase II carboxy-terminal domain kinase activity	MF	0.001	Up	GO:0008353	*CDK8/CDK12*
Negative regulation of TOR signaling	BP	0.004	Up	GO:0032007	*FLCN/UBR2*
Cellular amino acid metabolic process	BP	0.005	Up	GO:0006520	*DDC/KBL*
Cyclin-dependent protein serine/threonine kinase activity	MF	0.006	Up	GO:0004693	*CDK8/CDK12*
Guanyl-nucleotide exchange factor activity	MF	0.03	Up	GO:0005085	*FLCN/TRIO*
Cytoskeleton organization	BP	0.03	Up	GO:0007010	*RHO1/MAST1*
Fibrillar center	CC	0.03	Up	GO:0001650	*ZFP58/CDK12*
pyridoxal phosphate binding	MF	0.03	Up	GO:0030170	*DDC/KBL*
Ribose phosphate diphosphokinase complex	CC	0.007	Up	GO:0002189	*PRPS1*
Extracellular region	CC	0.02	Down	GO:0005576	*PA21B/SNO1/APOD/EXGB*
Transporter activity	MF	0.001	Down	GO:0005215	*APOD/SYPL1/MYP2*
Cholesterol binding	MF	0.002	Down	GO:0015485	*APOD/MYP2*
Axoneme	CC	0.003	Down	GO:0005930	*GAS8/BBS1*
Ciliary basal body	CC	0.009	Down	GO:0036064	*GAS8/BBS1*
Lamellipodium	CC	0.02	Down	GO:0030027	*ARPC3/PTN13*
Brain development	BP	0.03	Down	GO:0007420	*APOD/GAS8*
Cleavage in ITS2	BP	0.004	Down	GO:0000448	*NOL9*
Glycerol dehydrogenase [NAD+] activity	MF	0.004	Down	GO:0008888	*ADH1*
Alkaloid metabolic process	BP	0.004	Down	GO:0009820	*SNO1*
mTOR signaling pathway	KEGG	0.001	Up	KO:04150	*ULK3/RHO1/FLCN*
Antifolate resistance	KEGG	0.001	Up	KO:01523	*MRP2/MRP2*
Sphingolipid signaling pathway	KEGG	0.007	Up	KO:04071	*RHO1/MRP2*
24 h post exposure to salinity shock (222 non-overlapping DEGs in total)					
Plasma membrane	CC	0.001	Up	GO:0005886	*SIAH1/PIM1/RBGPR/C2CD5/CADN/CDIPT/DPOE1/NID2/ATPB1/PRC1/LIMS2*

					/TRPA1/IGF1R/MOT12/MFSD5/ENTP6/MYO1G/UNC5B/S12A9/JMJD6
Integral component of membrane	CC	0.03	Up	GO:0016021	*CSMD1/SSRD/CADN/CDIPT/S22A7/UBAC2/P3/TRPA1/PEN2/TM2D1/IGF1R/XYLT/MOT12/MFSD5/ENTP6/UNC5B/CHSTE/NOX5/S12A9/PERT*
Calcium ion binding	MF	0.005	Up	GO:0005509	*C2CD5/CADN/NID2/CAB45/ESYT2/NOX5/PERT*
Identical protein binding	MF	0.02	Up	GO:0042802	*SIAH1/ASH1/RNF4/PRC1/ESYT2/CASC3*
Ubiquitin-protein transferase activity	MF	0.02	Up	GO:0004842	*SIAH1/RNF4/FBX30/BIRC3/TRIM2*
Apoptotic process	BP	0.04	Up	GO:0006915	*SIAH1/PIM1/UNC5B/NOX5/THOC6*
Cytokinesis	BP	0.002	Up	GO:0000910	*PRC1/KI13A/NOX5*
rRNA binding	MF	0.002	Up	GO:0019843	*RPF1/RS4X/RM16*
Cell surface receptor signaling pathway	BP	0.01	Up	GO:0007166	*TSN31/BIRC3/JMJD6*
Intracellular ribonucleoprotein complex	CC	0.02	Up	GO:0030529	*RS4X/CASC3/JMJD6*
Nucleus	CC	0.002	Down	GO:0005634	*FOXG1/CLCA2/ZG17/ZFP28/NSF1C/DIDO1/PAK2/LARK/MCE1/SAP30/ZN207/NAA20/ZN317/METL4/ZN33B/TRI23/SQD/ABRU/ABHEB/TUT4/PSMD4/ZNF28/NUD16/HDAC3/CDK7/PLAG1/ASCC1/SNPC3/ATE1/RAD51/ZN155/AKR/SMCE1/ZN112/XCP1/ZFHX3/HSP70/PRD16/SRPK2/DMAD/SLF1/ADK/S18L2/SENP7*
Nucleoplasm	CC	0.01	Down	GO:0005654	*NSF1C/DIDO1/GIT2/DCA11/PSMD4/ZNF28/NUD16/PP6R3/S40A1/PLAG1/RAD51/AKR/SMCE1/ZN112/ZFHX3/GPN1/PRD16/SRPK2/RAD1*
Transcription, DNA-templated	BP	0.04	Down	GO:0006351	*FOXG1/ZG17/ZFP28/DIDO1/SAP30/ZN317/ZN33B/ABRU/ZNF28/HDAC3/PLAG1/ASCC1/SNPC3/ZN155/AKR/ZN112/ZFHX3/PRD16/S18L2*
Transcription factor activity, sequence-specific DNA binding	MF	0.04	Down	GO:0003700	*FOXG1/ZFP28/ZN317/ZN33B/ABRU/ZNF28/PLAG1/ZN155/ZN112/PRD16*
Golgi membrane	CC	0.03	Down	GO:0000139	*CHSTB/STX5/GTR1/TRI23/PP6R3/SEC20/GLT35*
Identical protein binding	MF	0.03	Down	GO:0042802	*GTR1/G6PD1/TRI23/PSMD4/NUD16/RAD51*
RNA polymerase II core promoter proximal region sequence-specific DNA binding	MF	0.02	Down	GO:0000978	*ZNF28/PLAG1/AKR/SMCE1*

Precatalytic spliceosome	CC	0.01	Down	GO:0071011	*LARK/SQD/HSP70*
Chromatin	CC	0.03	Down	GO:0000785	*SQD/WGE/RAD51*
Spindle	CC	0.04	Down	GO:0005819	*DIDO1/ZN207/PIN4*
Parkinson's disease	KEGG	0.004	Up	KO:05012	*UBB/QCR7/PPIA*
RNA transport	KEGG	0.005	Up	KO:03013	*THOC6/NUP85/CASC3*
Huntington's disease	KEGG	0.007	Up	KO:05016	*QCR7/PPIA/DCTN2*
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	KEGG	0.001	Up	KO:00532	*XYLT/CHSTE*
Acute myeloid leukemia	KEGG	0.009	Up	KO:05221	*PIM1/PANG1*
Bile secretion	KEGG	0.010	Up	KO:04976	*S22A7/ATPB1*
Cardiac muscle contraction	KEGG	0.011	Up	KO:04260	*QCR7/ATPB1*
Inositol phosphate metabolism	KEGG	0.015	Up	KO:00562	*YRBE/CDIPT*
Central carbon metabolism in cancer	KEGG	0.003	Down	KO:05230	*P55G/GTR1/G6PD1*
Thyroid hormone signaling pathway	KEGG	0.002	Down	KO:04919	*P55G/HDAC3/GTR1*

Adult survival decreased by 44±7% in response to 10 min exposure to handling stress. The average mortality reported by Jepsen et al. (2007) was 17±1% per day,which was considered to result from handling stress in adult individuals of *A. tonsa* every 12 h during their study. In comparison, other studies have reported constant daily mortality ranging from 5 - 10% (Drillet et al., 2014; Medina and Barata, 2004; Nilsson et al., 2017). The observed handling stress effect on survival in the present study is considered very high and indicative of impacts of handling stress for adult individuals of *A. tonsa*. Handling of adult copepods should therefore be done quickly in order to minimize the imposed stress in contrast to nauplii that does not seem to be affected on survival.

In comparison to handling stress, salinity shock resulted in a slightly higher adult survival (29±5%) after 10 min of exposure. Calliari et al. (2008) found that an abrupt reduction in salinity from S=35 to S=4 resulted in 31% decrease in survival, which is comparable to the decrease of 22 - 35% observed in this study.

Survival 24 h after exposure to 10 min handling stress and salinity shock had declined 45±1.7% and 51±1.6%, respectively. For handling stress, this was only an additional decline of ~7% in comparison to 15 min post exposure. For the salinity shock, the post-exposure period of 24 h was more lethal than for handling stress, with an additional decline of ~22%. This suggests that handling stress results in a higher instant mortality in comparison with salinity shock, while salinity shock is more lethal over an extended period post-exposure. Some of the instant mortality may be due to the physical damages observed for about one-third of the individuals, of which the majority was categorized as dead. In addition to the elevated mortality, the higher number of DEGs suggests that salinity shock is more stressful in comparison to handling.

In order to replicate the impact of "collection stress" and laboratory handling stress on *A. tonsa*, it was important to establish a baseline for copepods that were not exposed to handling stress. Since this is the first study examining impacts of handling stress at the transcriptome-wide level, there is no available information on the time course of the copepods' responses after handling stress. To avoid this as a bias in our

experiments, we cultured *A. tonsa* from eggs to the desired life-stages without disturbing the copepods.

For field studies, it is often impossible to know how long the copepods may have been in contact with a plankton net during collection and before preservation (e.g., Mack et al., 2012). According to the field guide by Goswami (2004), it is recommended to collect zooplankton by towing a plankton net at slow speed (1.5 – 2.0 knots) for 5-10 min. Additional collection time is used in pulling-in the plankton net and preparing the samples for preservation. The mortality of field-collected copepods, however, have been shown to range from 0 – 90% for visual discrimination and 13 – 37% for neutral stained copepods (Elliott and Tang, 2009). This is within the range of the survival decrease observed in the present study (44±7%) and suggests that observed mortality in field-collected copepods may be the result of handling stress. For laboratory studies, copepods are usually exposed to plankton net screens for shorter periods. But often other tools, like pipettes and tweezers, are used for transferring the copepods, which also can result in physical damage and stress.

Based on the observed mortalities, we chose the exposure time of 10 min for each stressor, which is within the range of handling time described in the literature, althoughstill so extreme that we were certain that we would induce a transcriptional response (Elliott and Tang, 2009; Mack et al., 2012).

In addition to the uncertainty regarding the magnitude and exposure time of the stressor that would induce a response at the transcriptional level, it was not clear when to expect a response post-exposure as well.

We found only two (1 up- and 1 down-regulated) and seven (6 up- and 1 down-regulated) DEGs 15 min after exposure to handling stress and salinity shock, respectively (Fig. 5, Table 3). None of these DEGs were in common between the treatments or have previously been used as transcriptional biomarkers. Handling stress resulted in 276 DEGs and salinity shock in 396 DEGs 24 h after exposure. Of these, 174 DEGs (112 up- and 62 down-regulated) were overlapping. The up-regulated expression of these genes may provide general protection against multiple stressors. However, the time period following exposure clearly has a significant impact on whether there is a detectable and measureable stress response.

The one up-regulated DEG 15 min after exposure to handling, *Inositol-Pentakisphosphate 2-Kinase* (*IPPK*), was assigned two BPs (melanosome transport and determination of left/right symmetry). *IPPK* in yeast are involved with the transcriptional regulation in response to environmental and nutritional changes; in plants, it is involved in stress signaling, and in mouse embryonic development (e.g. Tsui and York, 2010). The role of *IPPK* is, thus, very diverse among species and therefore the enriched BPs seems inexplicable. In *A. tonsa*, *IPPK* may have a role in initiating a transcriptional response towards handling stress due to its early up-regulation.

The gene product of the down-regulated *SID1 Transmembrane Family Member 1* (*SIDT1*) is involved in RNA-interference (RNAi), by transporting dsRNA across cellular membranes (e.g.,Whangbo et al., 2017). The down-regulation suggests that gene silencing of RNAi inhibited genes are being removed, which allows transcription.

The majority of enriched GO terms for up-regulated DEGs 15 min post exposure to salinity was related to protein transport, Endoplasmic Reticulum (ER), and protein homeostasis. This is an indication of ER stress, which typically will induce the unfolded protein response (UPR) in order to improve the imbalance between protein load and folding capacity of the ER (Hetz and Papa, 2017; Hori et al., 2006).

Enrichment of GO terms related to metabolic processes indicated the need for cellular energy and is in agreement with the observation by Calliari et al. (2006) that *A. tonsa* modulates its energy balance in relation to salinity stress. The enrichment of vitamin digestion and absorption (KO, Table 4) for *MAP kinase-interacting serine/threonine protein kinase 2* (*MAPK*) could reflect a need for energy. *MAPK* is, however, also induced in response to environmental stress as a part of a signaling cascade, hence the two signaling pathways in Table 4 (Waskiewicz, 1997).

The KEGG enrichment was done using general KO-terms, which are related to model organisms, e.g., human and mouse. The enriched terms and the actual functions may therefore differ in relation to copepods.

Five of the overlapping DEGs 24 hr post exposure to handling stress and salinity shock, were enriched for the BP, neurogenesis. These include *Histone deacetylase 1* (*HIDAC1*), *serine/threonine-protein phosphatase 4 regulatory subunit 2* (*PP4R2*), *helicase domino* (*DOM*), *longitudinals lacking protein* (*LOLA3*) and *selenide, water dikinase* (*SPS1*). Even though theyare enriched in the GO-term, neurogenesis, it is noteworthy that only *LOLA3* is directly linked to neurogenesis (e.g., Goeke et al., 2003). The gene product resulting from *HIDAC1* is mainly a regulator of gene expression for other genes, responsible for histone de-acetylation (e.g., Kelly and Cowley, 2013). *DOM* is, like *HIDAC1,* also responsible transcriptional regulation by chromatin remodeling (Sif, 2004). Especially *HIDAC1* is also enriched for terms related to chromatin remodeling, like chromatin (CC) and transcription corepressor activity (MF). *PP4R2* has functional roles in cell development, differentiation, apoptosis, tumor progression and DNA-repair (e.g., Liu et al., 2012; Nakada et al., 2008; Shui et al., 2007).

In addition to being enriched in GO-terms related to transcriptional regulation, oogenesis is enriched for *HDAC1*, *DOM*, *Ecdysone-induced protein 74EF isoform B* (*E74EB*) and *beta-1,3-galactosyltransferase BRN (BRN)*. Both *E74EF* and *BRN* are involved in oogenesis (Goode et al., 1996; Paul et al., 2005). When exposed to stressful conditions, energy of an individual tends to be reallocated from fecundity and growth to survival mechanisms (de Nadal et al., 2011; López-Maury et al., 2008). Thus, egg production of copepods decreases when the surrounding environment is sub-optimal (Calliari et al., 2006; Peck and Holste, 2006). The up-regulation of oogenesis may indicate that homeostasis in *A. tonsa* has been restored to such extent that there is energy for egg production.

Even though fecundity-related mechanisms were enriched for up-regulated overlapping DEGs 24 h post exposure to handling stress and salinity shock, stress related mechanisms were also present among the enriched terms (Table 5, S3a). This includes cellular response to DNA damage stimulus, ubiquitin protein ligase binding and chaperone-mediated protein folding (Table 5).

Heat shock proteins (*hsps*), especially *heat shock protein 70kDa* (*hsp70*), are frequently used as a transcriptional indicators of stress in copepods (Aguilera et al., 2016; Chan et al., 2014; Lauritano et al., 2011; 2016; Petkeviciute et al., 2015; Rahlff et al., 2017; Rhee et al., 2009; Smolina et al., 2016; Tartarotti and Torres, 2009; Voznesensky et al., 2004). We found significant, but small, down-regulation of *hsp70* in response to salinity shock 24 h after exposure (S1a). *Heat shock cognate 71kDa* (*hsc70*), which is a member of the hsp70 family, was slightly up-regulated for handling stress and salinity shock 24 h after exposure (S1a).

Aruda et al. (2011) found that the heat shock proteins, *hsp70a*, *hsp21* and *hsp22*, had significantly higher expression 3 h after handling with a plankton net, but did not find any significant differences 2 h after exposure. Rahlff et al. (2017) found significant changes in expression of *hsp70* in response to handling, which was reduced to negligible levels after 24 h. These prior studies suggest that the expression of *hsp70* peaks within 24 h after stress exposure, and may explain why we did not observe an increase in expression level of this gene 24 h after handling stress. *Hsp70* responses in relation to other stressors (e.g., temperature) seem to be in agreement with this explanation. Petkeviciute et al. (2015) found a 63.8 fold increase in *hsp70* transcripts for *A. tonsa* after a 45 min exposure to 30°C. This corresponds with the findings of Rahlff et al. (2017), where a heat shock of 28°C for 3 h resulted in significant up-regulation of *hsp70*, which was measurable after 30 min and peaked with a 185-fold increase after 1.5 h. A smaller peak of 60.4 fold increase remained 4 h after the heat shock (Rahlff et al., 2017). The peak in *hsp70* expression that occurred a few hours after exposure, which subsequently declined, could explain our findings. In general, the expression levels observed here were low, implying that *hsp70* and a number of other genes may show peak up-regulation within 24 h after exposure to stressors.

The down-regulated *hsp70* 24 h post exposure to salinity was enriched for the nucleus (CC, Table 6), precatalytic spliceosome (CC, table 6), presynapse (S3a), late endosomal microautophagy (BP, S3a), cellular response to topologically incorrect protein (BP, S3a), and perichromatin fibrils CC, S3a). Many of these terms are stress-related, and could have included additional GO-terms that are relevant in relation to *hsp70* "chaperone mediated protein folding" and "*de novo* protein folding" (S3a).

It is noteworthy that aldehyde-dehydrogenases (*ALDH2* and *ALDH7A1*), ubiquitin – and related genes (e.g., *UBP25*, *FANCL*, *UBR2, UBR4*, *UBE2C*, Table 5, S1a, S3a), which have been used in copepods as transcriptional biomarkers, are among the overlapping DEGs 24 h post exposure to handling stress and salinity shock (e.g., Lauritano et al., 2011; 2016). Biomarkers should, thus, be carefully selected to avoid that handling cause artifact in the analysis of gene expression.

In sum, handling stress clearly affects both biomarkers and transcriptome-wide patterns of differential gene expression of *A. tonsa,* and these stress responses probably take place within 24 h after exposure to a stressor. Some of the differentially expressed genes were in common between the handling stress and salinity shock treatments, suggesting that these may play an important role in protection against

multiple stressors. Due to the small, but significant differences in expression levels of some of the commonly used biomarkers, these genes should be used with caution in stress-related studies, since they potentially peak within 24 h after exposure.

The limited response at the transcriptional level 15 min following exposure to handling stress suggests that organisms collected in plankton tows of short duration and preserved immediately are likely to exhibit transcriptional profiles that represent their *in situ* physiological state (e.g., Häfker et al., 2017).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

B.N., P.M.J., A.B and B.W.H. conceived the study; B.N., P.M.J., A.B., and B.W.H. designed the experiments; B.N., P.M.J. and A.B. collected and analyzed the data; B.N. wrote the paper; B.N., P.M.J., A.B. and B.W.H. contributed substantially to interpreting the data and developing the manuscript, and take full responsibility for the content of the paper.

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Data availability

RNA-Seq data available in the NCBI Sequence Read Archive with the bio-project accession number PRJNA407266.

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MANUSCRIPT III: **Timing of quiescence determines viability of eggs from the calanoid copepod, *Acartia tonsa* (Dana)**

Similar to 41 other species of calanoid copepods, *Acartia tonsa* is capable of inducing embryonic quiescence in response to unfavorable conditions in the surrounding environment. This manuscript provides a detailed description of subitaneous development and quiescence in *A. tonsa* eggs.
The manuscript demonstrates that the ecdysone-signaling pathway is important during subitaneous development. Furthermore, it was demonstrated that the duration of quiescence determines the viability of the eggs.

This work provides new knowledge about copepod embryonic development and dormancy.

Timing of embryonic quiescence determines viability of embryos from the calanoid copepod, *Acartia tonsa* (Dana)

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ABSTRACT

Like 41 other calanoid copepods, *Acartia tonsa*, are capable of inducing embryonic quiescence when experiencing unfavorable environmental conditions. The ecdysone-signaling cascade is known to have a key function in developmental processes like embryogenesis and molting of arthropods, including copepods.We examined the role of *ecdysteroid-phosphate phosphatase* (*EPPase*), *ecdysone receptor* (*EcR*), *ß fushi tarazu transcription factor 1* (*ßFTZ-F1*), and the *ecdysteroid-regulated early gene E74* (*E74*), which represent different levels of the ecdysone-signaling cascade in our calanoid model organism. Progression of embryogenesis was monitored and hatching success determined to evaluate viability. Embryos that were induced quiescence before the gastrulation stage would stay in gastrulation during the rest of quiescence and exhibited a slower pace of hatching as compared to subitaneous embryos. In contrast, embryos developed further than gastrulation would stay in gastrulation or later stages during quiescence and showed a rapid pace in hatching after quiescence termination. Expression patterns suggested two peaks of the biological active ecdysteroids, 20-hydroxyecdysone (20E). The first peak of 20E was expressed in concert with the beginning of embryogenesis originating from yolk-conjugated ecdysteroids, based on *EPPase* expression. The second peak is suggested to originate from *de novo* synthesized 20E around the limb bud stage. During quiescence, the expression patterns of *EPPase*, *EcR*, *ßFTZ-F1,* and *E74* were either decreasing or not changing over time. This suggests that the ecdysone-signaling pathway play a key role in the subitaneous development of *A. tonsa* embryogenesis, but not during quiescence. The observation is of profound ecological and practical relevance for the dynamics of egg banks.

Keywords: ecdysteroids, copepods, embryogenesis, subitaneous, quiescence

INTRODUCTION

Copepods are of high ecological importance by linking energy and matter from phytoplankton to higher trophic levels of the pelagic marine food web [1]. The calanoid copepod, *Acartia tonsa*, inhabits estuaries and nearshore environments of temperate waters [2]. Besides a high tolerance towards environmental changes, *A. tonsa* are like 41 other calanoids capable of coping with seasonality and stressful conditions by producing dormant embryos where embryogenesis is on a halt [3]. Subitaneous embryos, hatch within a few days after oviposition when the surrounding conditions are optimal. When conditions are sub-optimal, the embryos can survive by undergoing dormancy, which comprises following three types: quiescence, which is defined as retarded development, oligo-pause which is delayed hatching embryos and diapause, where development is arrested [4–7].

The dormant embryos sink to the bottom where they get buried and accumulate in the sediment egg bank. Depending on the type of dormancy and sediment conditions, the embryos can survive for years. When the sediment is being disturbed and dormant embryos return to the water column under favorable conditions, they will hatch and recruit new copepods to the pelagic population [5, 8].

Even though copepods are ecological important not much is known about the underlying embryonic mechanisms during subitaneous development and quiescence. Most studies are dealing with the post-embryonic development of calanoid copepods, but only a few studies concern the embryonic development in further details [9, 10].

Ecdysteroids is a group of polyhydroxylated sterols that in arthropods mediates embryonic development, molting, metamorphosis, and adult development by stimulating curticular protein (CP) production [11, 12]. Embryonic ecdysteroids are secreted from ovarian follicle cells and converted into conjugates with yolk proteins [13–17]. During embryogenesis, the enzyme, ecdysteroid-phosphate phosphatase (EPPase), hydrolyzes conjugated ecdysteroids into free-form as the yolk-proteins continuously degrade [18, 19]. In addition, embryonic ecdysteroids are also suggested to be *de novo* synthesized enzymes encoded by a set of genes called the Halloween genes [20].

The biological active ecdysteroid, 20-hydroxyecdysone (20E) will upon interaction with a heterodimer receptor complex consisting of the ecdysone receptor (EcR) and ultraspiracle (USP), initiate a cascade of ecdysteroid responsive genes that ultimately will stimulate embryonic molting by targeting the expression of CP genes [12, 21–23]. Embryonic molting is the deposition of embryonic cuticles [24, 25].

Following interaction between 20E and the EcR/USP complex, the expression of *fushi tarazu (ftz) transcription factor 1* (*FTZ-F1*) will be stimulated, and target down-stream ecdysone responsive genes, including the *early genes 74* and *75* (*E74*, *E75*) and the *Broad-Complex* [12]. There are produced two isoform transcripts from *FTZ-F1* that differ in the N-terminal; *αFTZ-F1* and *ßFTZ-F1* [26]. The *αFTZ-F1* isoform is transcribed in relation to *ftz* expression in early embryogenesis [27]. The *ßFTZ-F1* are, on the other hand, expressed in later stages of embryogenesis [28, 29]. The *ßFTZ-F1* expression is timely restricted during development and have an important role in late embryogenesis and later molting processes by regulating the ecdysteroid-

responsive genes, including E74 [12, 30]. In addition to *E74*, and the other ecdysteroid responsive genes, *ßFTZ-F1* stimulates expression of the Halloween genes, *Phantom* and *Disembodied*, involved in ecdysteroid biosynthesis [31, 32].

The biological active 20E in insects have been shown to be able to abrupt the dormant state, diapause [33, 34]. This suggests that ecdysteroids have a function during the subitaneous development as well as dormant states of embryogenesis.

In the present study, the embryogenesis of *A. tonsa* was examined during subitaneous development and quiescence. Progression of embryogenesis was monitored by visualization of DAPI-stained embryos over time and hatching success was estimated to determine viability. Gene expression of *EPPase*, *EcR*, *ßFTZ-F1,* and *E74* were analyzed at key-times during embryogenesis since they represent four different levels of the ecdysone-signaling cascade leading to embryonic molting and development. The significance of the timing of embryonic quiescence is discussed with reference to natural- and culture egg banks.

RESULTS

Hatching success

After 24 h of subitaneous development, 9.0 ± 2.9% (mean ± SD) of the embryos had developed into fully hatched nauplii. The hatching success after 48 h, 72 h, and 96 h were 73.7 ± 2.9%, 87.2 ± 3.3%, and 89.6 ± 4.1%, respectively (Table 1). For embryos undergoing 14 d of quiescence initiated maximum 1 h after oviposition had after 24, 48, 72, and 96 h following hatching success: 0.6 ± 0.7%, 49.8 ± 3.6%, 63.9 ± 4.4%, and 82.7 ± 2.8% (Table 1). Embryos developed to the LB stage (32 h), followed by 14 d of quiescence, exhibited hatching success of 81.0 ± 2.9%, 86.9 ±1.9 %, 87.8 ± 2.8%, and 89.7 ± 2.5 % after 24, 48, 72, and 96 h, respectively (Table 1). The number of embryos was consistent with the resulting hatched nauplii.

Table 1. *Hatching success (%) of Acartia tonsa after subitaneous development, quiescence for 14 d, and subitaneous development for 32 h followed by 14 d of quiescence. Hatching success is given in percentage hatching (mean ± SD) after 24, 48, 72 and 96 h.*

Development	24 h	48 h	72 h	96 h
Subitaneous development	9 ± 2.9 %	73.7 ± 2.9 %	87.2 ± 3.3%	89.6 ± 4.1%
Quiescence, 14 d	0.6 ± 0.7%	49.8 ± 3.6%	63.9 ± 4.4%	82.7±2.8%
Subitaneous development, 32 h, Quiescence, 14 d	81.0 ± 2.9%	86.9 ±1.9 %	87.8 ± 2.8%	89.7 ± 2.5 %

DAPI staining

Embryogenesis was classified into 14 stages: 1 cell (S1), 2 (S2), 4 (S3), 8 (S4), 16 (S5), 32 (S6), (S7) and 128 cells to blastula (S8; in blastula, the cells are arranged in multiple rows), gastrulation (G), organogenesis (O), limb bud (LB), appendages appear (AA), early nauplii (EN) and final nauplii (FN). Only embryos with proper staining were used for stage determination.

During subitaneous development, the majority of embryos were present as S1 (52.4%, Fig. 1) and S2 (42.9%, Fig. 1) 1 h after oviposition. The brighter nucleus was faintly appearing approximately in the middle of the cell. The interference from the surrounding yolk made the visualization a bit unclear. Brighter areas observed in some of the embryos could indicate the polar bodies, but this was not studied further. The cleavage resulting in S2 was typically appearing asymmetric with the one cell larger than the second. From 1 to 4 h after oviposition, the embryogenesis gradually develop into S8 (majority 43.5%, Fig. 1) by sub sequential cell cleavages. From 5 h after oviposition, the majority of the embryos start to enter G (Fig. 1). The embryos were then present mainly in G, O, and LB until 32 h after oviposition (Fig. 1). Hereafter the embryogenesis continued gradually until hatching around 48 h for most nauplii (Fig. 1). Development during quiescence was slower than for subitaneous eggs (Fig. 2). From 1 h (S1, S2) to 36 h after oviposition following induced quiescence the embryos developed from S1 to S8 (Fig. 2). Initial G was induced around 40 h, and the developmental progression stalled in G 48 h – 3 d after induced quiescence (Fig. 2).

Embryos undergoing subitaneous development for 32 h followed by 14 d quiescence were also DAPI stained (n = 28) based on Fig. 1 and 2. The staining exhibited that the embryos present were in following stages G (3.6%), O (7.1%), LB (32.1%), AA (21.4%), EN (21.4%) and FN (14.3%). As a control, embryos from the same batch, maximum 1 h after oviposition, had induced quiescence for 14 d followed by DAPI-staining (n=19). All of the control embryos (100%) were in the G. Of the non-hatching embryos after 14 d of quiescence was 26.3% in G, 15.8% in O, 26.3% in LB, 21.1% in AA, and 5.6% of both EN and FN (n=19). For non-hatching embryos with subitaneous development up till 32 h followed by 14 d of quiescence had following stage distribution; 25.0% in G, 16.7% in O, 25.0% in LB, 8.3% in AA, 16.7% EN, and 8.3% FN (n=12). The ~2.5-month old cold-stored stained embryos (n=31) were present in G (16.1%), O (12.9%), LB (22.6%), AA (19.4%), EN (25.8%), and FN (3.2%).

Only embryos that were properly stained were used for stage-determination. Some of the embryos exhibited proper DAPI staining only on the surface corresponding to bacteria and algae on the chorion. These were either dead or failed to be stained for some reason. Other embryos showed sign on punctuations and leakage. These embryos were all excluded from the study.

Gene expression analysis

A one-way ANOVA was carried out on gene expression (log2FC) over time (h) for *EPPase*, *EcR*, *ßFTZ-F1* and *E74* during subitaneous development and quiescence.

The genes, *EPPase*, *ßFTZ-F1* and *E74,* had significant changes in gene expression over time for both the subitaneous and quiescent state (Table 2, Fig. 3). *EcR* only exhibited significant change over time for subitaneous development (Table 2, Fig. 3). Tukey's HSD post hoc tests were carried out to estimate at which times gene expression differed significantly for the 4 genes.

The expression of *EPPase* at 1 and 4 h, during subitaneous development, both differed significantly from 5, 12, 16, 32 and 48 h ($p < 0.05$, Fig. 3A). In addition,

EPPase expression differed significantly after 48 h in comparison to 7 h ($p < 0.05$, Fig. 3A). In the quiescent state, the gene expression of *EPPase* was significantly lower at 7 d than 1, 12, 32 and 48 h ($p < 0.05$, Fig. 3B).

EcR did not exhibit any significant changes in gene expression over time during quiescence (Fig. 3D). But 16 h of subitaneous development differed significantly from 1, 4, 7 and 32 h ($p < 0.05$, Fig. 3C).

During the subitaneous development of *A. tonsa*, the expression of *βFTZ-F1* was significantly different at 32 h in comparison to 4, 5, 7, 12 and 16 h ($p < 0.05$, Fig. 3E). The 48 h did not differ from 32 h for *βFTZ-F1* but were significantly different from 12 and 16 h ($p < 0.05$, Fig. 3E). Furthermore, the 1 h of *βFTZ-F1* differed significantly from 12 h ($p < 0.05$, Fig. 3E). The expression of *βFTZ-F1* was significantly lower after 7 d of quiescence in comparison to 1, 4, 5, 7, 12 and 32 h ($p < 0.05$, Fig. 3F).

E74 had a significant change in gene expression at 1 and 4 h in comparison to 5, 7, 12 and 16 h ($p < 0.05$, Fig. 3G) during subitaneous development. Gene expression of *E74* at 32 h was significantly different from 5, 7, 12, 16 h, and 48 h ($p < 0.05$, Fig. 3G). During quiescence, the expression of *E74* at 1 h was significantly higher in comparison to the other times, except 7 h ($p < 0.05$, Fig. 3H). Furthermore, the expression of E74 was significantly lower at 7 d quiescence in comparison to 1, 4, 5, 7, 12 and 32 h ($p < 0.05$, Fig. 3H).

DISCUSSION

To examine the role of ecdysteroids during subitaneous development and quiescence of *A. tonsa* embryos, we evaluated the hatching success, embryogenesis progression and gene expression of key genes involved in the ecdysone-signaling cascade.

The subitaneous development of *A. tonsa* resulted in the hatching of ~10% after 24 h. This increased to ~75% after 72 h followed by a plateau of ~90% after 48 and 96 h of subitaneous embryogenesis. Similar hatching patterns have been observed in studies using the same *A. tonsa* strain and temperature as in the present study [35]. For instance, one study reported a hatching of 55 % after 24 h and 90 % after 48 h, while another reported hatching of ~25% after 24 h and ~65% after 48 h [35, 36]. Regardless of the pattern leading to saturation, *A. tonsa* hatching reaches a plateau around 90% within 96 h [35–38].

Post-quiescent embryos exhibited a slower hatching pace in comparison to those with subitaneous development. Only about 1 % of the embryos hatched within 24 h, that slowly increased to ~83% after 96 h. The post-quiescent hatching pattern is, however, similar to [36], that increased to ~84% after 96 h.

Compared to the DAPI visualization of subitaneous embryogenesis, the developmental progress stalled at the G stage around 48 h, were it remained for the rest of the quiescence. The slower hatching pace post-quiescence suggests that the embryos have to ‘wake up’ their development from their G stage before continuing subitaneous development, which takes longer time than if embryos were present in later stages than G (i.e. 32 h subitaneous embryos followed by 14 d quiescence).

To challenge this, embryos undergoing subitaneous development for 32 h, corresponding to the LB stage, were induced quiescence for 14 d. The hatching of these embryos was rapid, with a success ~82% after 24 h, which also plateaued around 90% after 96 h. The staining of these embryos revealed that only G – or developmental stages beyond that up to FN were present. The staining of ~2.5-month cold-stored embryos had similar stage-distribution. This suggests embryos that have not reached G will develop to this stage during quiescence and stay there, which also was confirmed by the positive control from the same embryo batch (i.e. 14 d quiescence). If the embryos have progressed further than G during subitaneous development, the development will continue slowly towards FN, probably corresponding to the lower temperature conditions. These embryos will probably burn out of energy faster than embryos developing to and staying in G during quiescence.

For the present study, we examined embryos with an age of maximum ~2.5-months. In the literature, hatching of *A. tonsa* have shown to be ~70-80% after 3 and 11-months, ~40% after 12-months, and no viable embryos after 20-months of cold-induced quiescence [39]. The duration limit in relation to quiescence is suggested to be due to energy-depletion, where fatty acid pools for embryonic development are reduced over time [40, 41].

The observed ~10% non-hatching embryos were all present in G or exceeding developmental stages. The development of these are probably at a slower pace in comparisons to other embryos from the same batch and can be categorized as delayed-hatching [7].

The expression of *EPPase* at 1 and 4 h (S1-S8) differed from the rest of the subitaneous development, except at 7 h, which could be because of an outlier in the data. This suggests that *EPPase* have an important role in the beginning of *A. tonsa* embryogenesis. The expression after 1 and 4 h gradually decreased towards hatching. In the water-flee, *Daphnia magna*, *EPPase* expression have shown to be elevated during the first 6 hours of embryogenesis until the beginning of O [42]. This is comparable with the observed expression levels for *A. tonsa*, where *EPPase* is elevated just prior G. Gene knockout of *EPPase* have shown to result in the arrested development of *D. magna*, which confirms its essential role in early subitaneous development [42]. In the quiescent state, *EPPase* exhibited down-regulated expression after 1 h, which is consistent with the lack of *EPPase* activity observed during insect diapause [23, 42]. During insect diapause, embryonic ecdysteroids are mainly found in conjugated form, while subitaneous development has free- and conjugated forms coexisting, which could explain the low expression of *EPPase* [18, 35, 36].

EcR exhibited a decrease in expression from 1 to 16 h during subitaneous development, followed by a slight peak at 32 h. This could suggest a more profound peak in *EcR* expression between 16 and 32 h, that would be transcribed prior *ßFTZ-F1* and *E74*, which both peaked at 32 h [12].

Expression of *ßFTZ-F1* gradually decreased from 1 h to 12 h post-oviposition, followed by an increase that statistically peaked at 32 h corresponding to the LB stage. The 1, 4 and 48 h did not result in significant results, which could be due to

gradual changes in expression over time. The initial higher levels – and peak the of *ßFTZ-F1* during subitaneous development, probably indicate a previous rise in E20 titer [12].

The initial decrease corresponds to the observed levels of *EPPase* – suggesting that 20E initially are originating from yolk-conjugated ecdysteroids. Since there is no later corresponding a peak in *EPPase* expression, but in *ßFTZ-F1*, this could suggest that the 20E at that point are *de novo* synthesized by Cytochrome P_{450} enzymes encoded by Halloween genes [20, 23]. A similar expression pattern was observed for E74, but the decrease after 4 h until the peak at 32 h where, however, abrupt drop rather than a gradually decrease. The matching expression patterns of *ßFTZ-F1* and *E74* corresponds to that *ßFTZ-F1* stimulated the expression of *E74*, which in turn targets CP genes further down the ecdysone-signaling cascade [11, 12].

During subitaneous development of the silkworm, *Bombyx mori*, 20E are only present to a minor degree, but around G and O the levels increase rapidly [23]. In *A. tonsa*, this could be consistent with the observed peaks in the expression of *ßFTZ-F1* and *E74* that are observed around LB. The observed levels of *ßFTZ-F1* and *E74* are consistent with levels observed in other studies [12].

During quiescence, *EPPase*, *ßFTZ-F1,* and *E74* exhibited a decreasing pattern in

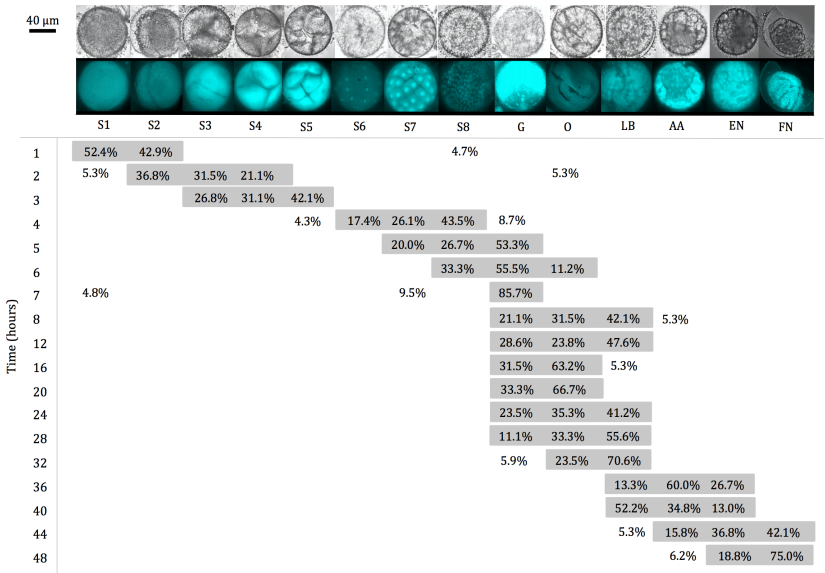

Figure 1. Percentages of embryonic stages during the subitaneous development of *Acartia tonsa*. On the top bright filter – and epifluorescent images can be seen for DAPI stained embryos of *A. tonsa*. Embryogenesis was divided into following stages: 1 cell (S1), 2 (S2), 4 (S3), 8 (S4), 16 (S5), 32 (S6), (S7) and 128 cells/blastula (S8), gastrulation (G), organogenesis (O), limb bud (LB), appendages appear (AA), early nauplii (EN) and final nauplii just before hatching (FN). For each of the developmental times, 26±2 embryos were used for stage estimation. Contrast and brightness were adjusted for publication.

expression with lowest levels at 7 d. The expression of *EcR* did not change significantly over time. This suggests that the ecdysone-signaling cascade, and thereby ecdysteroids, have a key role in the subitaneous development of *A. tonsa*, but not during quiescence.

In summary, we have for the first time demonstrated that embryos of our model calanoid species *A. tonsa* stay in G if the embryos prior have not been developed beyond that point in development. Embryos developed further than the G stage appeared to be present in later stages during quiescence. Hatching from embryos no older than 1 h before induction of quiescence happened at a slower pace in comparison to subitaneous embryos. This suggests that the embryos have to 'wake up' from quiescence in G before continuing embryogenesis. Embryos developed to around LB had 'instant' hatching within 24 h after the quiescent conditions were terminated, which support the findings of that younger embryos are present in G,

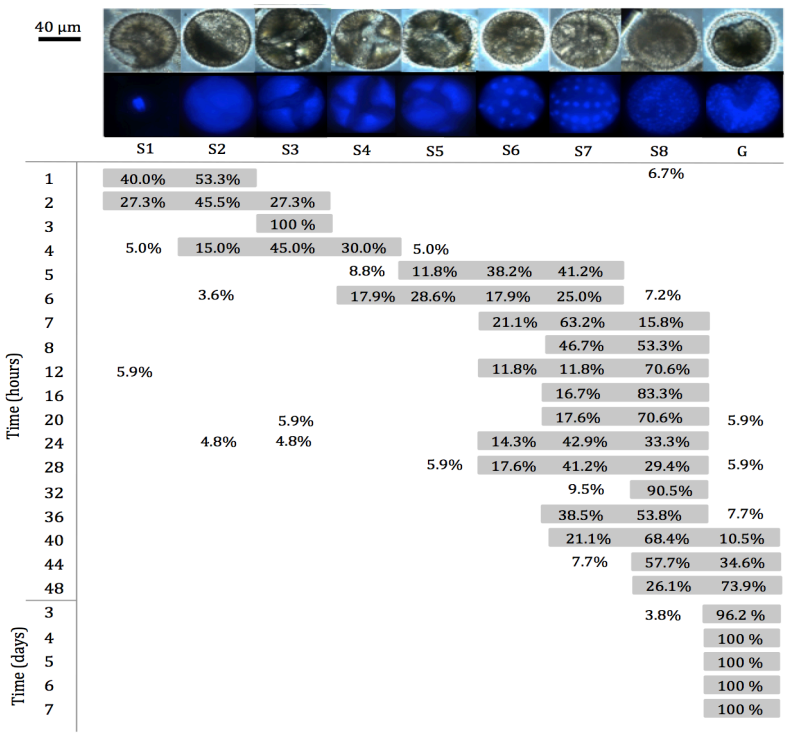

Figure 2. *Percentages of embryonic stages during quiescence of Acartia tonsa. On the top bright filter – and epifluorescent images can be seen for DAPI stained embryos of A. tonsa. Embryogenesis was divided into following stages: 1 cell (S1), 2 (S2), 4 (S3), 8 (S4), 16 (S5), 32 (S6), (S7) and 128 cells/blastula (S8), gastrulation (G). For each of the developmental times, 19±4 embryos were used for stage estimation. Contrast and brightness were adjusted for publication.*

while older are present in stages beyond that. The expression patterns of the four genes involved at different levels of the ecdysone-signaling cascade suggest two peaks in 20E titer. The first one at the beginning of embryogenesis, originating from yolk-conjugated ecdysteroids based on the *EPPase* expression. The second around the LB stage probably caused by a peak in *de novo* synthesized 20E, since there are no changes in expression of *EPPase* but of *βFTZ-F1*, *E74* and possible *EcR,* which are further in the signaling cascade.

Embryos undergoing dormancy constitute ecological and evolutionary reservoirs that are able to recruit new individuals to a pelagic population and ensure its survival during environmental change. Species capable of having egg banks of viable dormant embryos will have a better chance of survival during adverse conditions, in comparison to species without [43].

We suggest that time for initiating quiescence is of imperative significance for embryonic survival in natural egg banks vital for later re-inoculation of the pelagic after e.g. overwintering [44]. Moreover, it is an aspect of relevance for optimizing protocols for generating culture egg banks in relation to live feed products for marine fish hatcheries [45, 46].

MATERIALS AND METHODS

Cultures

The *A. tonsa* strain used for cultivation originated from Øresund (N 56°/E 12°; Denmark) and was isolated in 1981 (DFH-ATI, Støttrup et al. 1986). The strain has for 30 years been maintained under constant salinity, temperature and light conditions (salinity 32, 17°C, dim light) and fed on a diet consisting of the mono-algae, *Rhodomonas salina* (identity code: K-1487). Three copepod cultures were set up prior the experiments in order to provide statistical replicates. The cultures were kept in 60 L flat-bottomed polyethylene tanks at the same stable conditions as mentioned above in 40 L 0.2 μm-filtered seawater with gentle aeration. The copepods were fed *R. salina ad libitum* every day (>800 µg C L^{-1}; Berggreen et al., 1988), which was cultivated in 2.0 L round-bottom glass flasks with F2 media [49]. Cultivation took place under stable conditions at 17°C with CO_2 supply and light (PAR~80μE m^{-2} s^{-1}). All experiments were conducted with 0.2μm-filtered seawater with a salinity of 32.

Hatching success

Adult copepods isolated with a 400μm mesh was transferred to 1.0 L beaker glasses containing seawater in order to spawn. After 1 h of incubation, the adults were separated from the embryos using a 100μm mesh on top of a 60μm mesh. Embryos were transferred to 50 mm in diameter petri dishes and incubated at 16.9 ± 0.1°C (mean ± SD) for subitaneous development in 10 mL seawater. Embryos and nauplii were counted after 24, 48, 72 and 96 h in order to determine the hatching success. Subitaneous hatching success was performed with 6 replicates (624 ± 113 embryos per replicate, mean ± SD).

Based on the following DAPI visualization of embryogenesis, embryos were within 1 h after oviposition (n = 160 ± 24, 5 replicates) transferred to petri-dishes and incubated at 3.0 ± 0.2°C for 14 days to induce quiescence. Furthermore, additional embryos (n = 191 ± 26) were undergoing subitaneous development for 32 h at 16.9 ±

0.1°C before being incubated at 3.0 ± 0.2°C for 14 days for quiescence. Embryos and nauplii were following quiescence counted after 24, 48, 72 and 96 h to determine hatching success. The embryos that remained un-hatched after 96 h were selected and stained as described in the DAPI staining.

DAPI staining

Adult copepods from the three individual cultures were isolated with a 400μm mesh and transferred to 1.0 L beaker glasses containing 800 mL seawater with gentle aeration. The copepods were fed *R. salina* and incubated to spawn for 1 h in darkness at 16.9 ± 0.1°C (mean ± SD). The adults, and other life-stages were separated from the embryos by using a 100μm mesh on top of a 60μm mesh to collect the embryos. Embryos were transferred to 1.5 mL Eppendorf tubes containing seawater and incubated at 16.9 ± 0.1°C. Sampling was done each hour from 1 to 8 h, and for every fourth hours from 8 h to 72 h. The samples were centrifuged at 10.000 rpm for 30 seconds in order to pellet the embryos. Residual seawater was removed by pipetting and embryos were fixed by sodium tetraborate-buffered 4% formaldehyde in seawater, in order to stop the biochemical activities and increase mechanical strength [50]. The seawater used for dilution of the formaldehyde was the same as used for cultivation of the copepods in order to avoid the undesired effects of osmotic stress in the embryos. Embryos treated with the fixation solution were stored at 3 ± 0.2°C (mean ± SD) in darkness for a minimum of 24 h before further processing.

Embryos, from each developmental time-step, were stained with DAPI according to [51]. De-ionized water was used to cause osmotic stress to such a degree that it allowed the uptake of the fluorescent molecule into the embryo and the nuclei. Ethanol-rinsed embryos from each sample were transferred to a depression slide (n = 19 ± 2 eggs, mean ± SD) containing 100 µL of 10 µg/mL DAPI and then covered with a coverslip. The depression slides containing the treated embryos were incubated at 3 ± 0.2°C (mean ± SD) in darkness for 24 h.

Besides the progressive development during subitaneous and quiescent state, the non-hatching embryos described under hatching success, as well as ~2.5-months old cold-stored embryos, were also DAPI-stained as described here. The ~2.5-months old cold-stored eggs originated from the *A. tonsa* embryo-bank at Roskilde University harvested July the 25th, 2017 (stained October the 3rd, 2017).

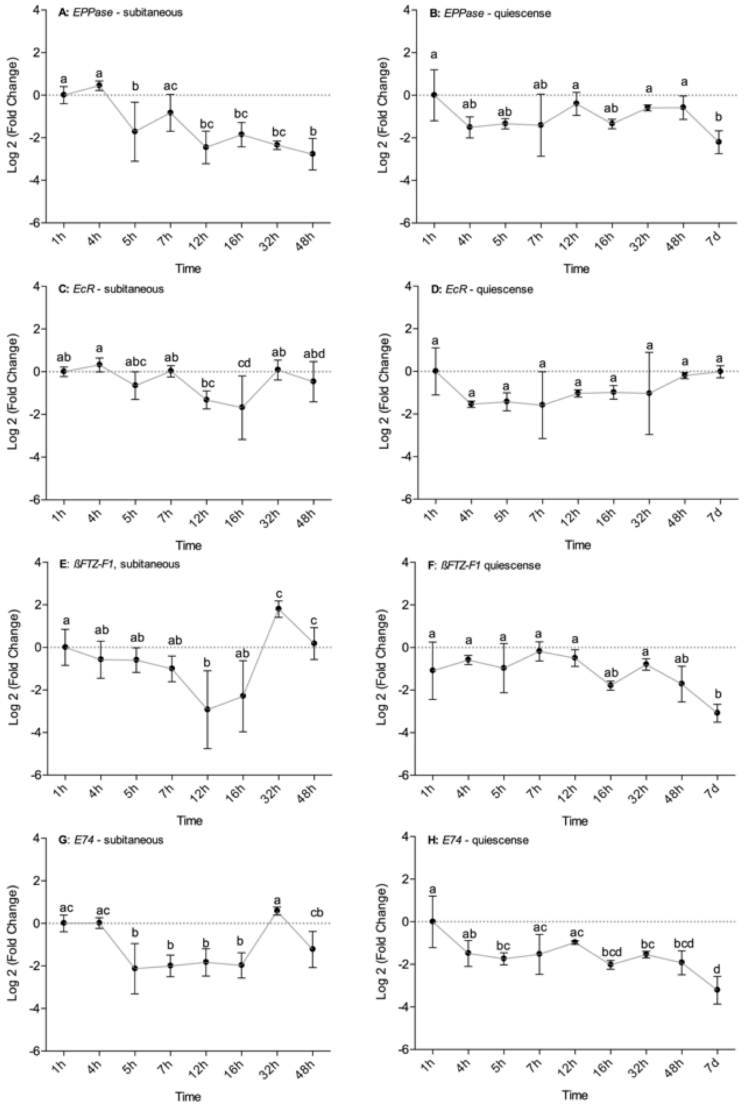

Figure 3. *Real-time quantitative PCR analysis of A) ecdysteroid-phosphate phosphatase (EPPase), subitaneous development; B) EPPase, quiescence; C) ecdysone receptor (EcR), subitaneous development; D) EcR, quiescence; E ß fushi tarazu transcription factor 1 (ßFTZ-F1), subitaneous development; F) ßFTZ-F1, quiescence; G) ecdysteroid-regulated early gene E74 (E74), subitaneous development; and H) E74, quiescence, of Acartia tonsa. For each of the selected developmental times, 4 biological replicates were collected. Each biological replicate contained 100 embryos of A. tonsa. The real-time quantitative PCR was performed with 3 technical replicates. Expression levels were normalized to the geometric mean of two stable reference genes, histone 3 (HIST) and ATP synthase (ATPS). Error bars represent standard deviation. Tukey HSD was used to test for significant differences in gene expression (log2 FC) over time (h). Different letters indicate statistically significant differences ($P < 0.05$). Statistics were conducted using R and the graph was generated in GraphPad Prism (ver. 6).*

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The subitaneous embryos were examined with a Nikon Eclipse Ti-U inverted microscope (Nikon, Nikon Instruments Europe B.V.) equipped with a 60X dry Plan Apo objective. The epi-illumination was made by a LED epifluorescence UV (365 nm) light source (CoolLED ltd, Andover, UK), using Nikon´s UV-2B filter set (excitation at 330 – 380; emission at >435nm). Images were taken by a DS-QiMc camera (Nikon, Nikon Instruments Europe B.V.) powered by NIS elements™ software package (Nikon, Nikon Instruments Europe B.V). Images were made of multiple focus layers to create full depth of field images by scanning images through the embryos. After assembly of multiple focus layers, the final image was de-convoluted using Photiosity (version 1.7.0, Rolf Geprägs, Hamburg, Germany) with a grid size of 25 pixels and 100 % noise control.

Quiescent embryos were examined with an Axio Vert.A1 FL inverted microscope with an LD EC Epiplan-Neofluar 50x/0.55 HD DIC M27 objective (Carl Zeiss, Welwyn Garden City, UK). Epi-illumination was provided by a LED epifluorescent UV (365 nm) HXP 120 C light source, using a DAPI filter (Carl Zeiss, Welwyn Garden City, UK). Images were taken using an AxioCam MRc digital camera and the AxioVision (ver. 4.8) analysis software by automatic Z-stack scanning the embryos through in 20 slides of 2 µm each (Carl Zeiss, Welwyn Garden City, UK). Convolution of the assembled images where processed in the same way as for the subitaneous embryos.

Table 2. *One-way ANOVA results: ecdysteroid-phosphate phosphatase (EPPase), ecdysone receptor (EcR), β fushi tarazu transcription factor 1 (βFTZ-F1), and ecdysteroid-regulated early gene E74 (E74). The subitaneous development had a degree of freedom (DF) of 7, while quiescence had a DF of 8.*

Gene	Subitaneous development		Quiescent state	
	F-value	***p-value***	***F-value***	***p-value***
EPPase	10.280	6.55e-06	3.840	0.00454
EcR	3.987	0.00502	1.636	0.165
βFTZ-F1	7.687	6.76e-05	6.168	0.000234
E74	11.380	2.73e-06	7.686	3.64e-05

Gene expression analysis

Based on the visualization of the embryonic development with DAPI-staining embryos were sampled for real-time quantitative PCR (qPCR) according to the developmental times given in S1. For each of the developmental-times quadruplicate samples, each containing 100 embryos, was collected, flash frozen and stored at -80°C until further processing.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). To ensure complete homogenization, embryos were first homogenized in 50 µL RLT buffer using disposable micro-pestles, after which additional 250 µL RLT buffer was added. The samples were vortexed for 5 sec and then processed according to the manufacturer's protocol, with a final elution volume of 30 µL in RNase-free

water. RNA concentration and purity were measured using a Nano-Drop 2000/2000c spectrophotometer (ThermoFisher Scientific Inc., USA). RNA integrity was tested on a denatured 1% agarose gel stained with ethidium bromide. Only samples with 280/260 absorbance's between 1.8 and 2.0 - and two distinct bands, corresponding to 28s and 18s rRNA, on the gel were used for further analysis.

50 ng RNA from each sample was transcribed into cDNA with the QuantiTect Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany) according to manufacturers' protocol with the gDNA wipe-out treatment for removal of genomic DNA. The qPCR primers that are listed in S2 were produced in Geneious® (ver. 9.1.6, Biomatters Ltd, New Zealand). Sequences of *ecdysteroid-regulated early gene E74* (*E74*), *ecdysone receptor* (*EcR*), *ecdysteroid-phosphate phosphatase* (*EPPase*), *β fushi tarazu transcription factor 1* (*βFTZ-F1*), *Histone 3* (*HIST*) and *ATP synthase* (*ATPS*) from arthropod species found in the NCBI database were used to BLAST against the *A. tonsa de novo* transcriptome (accession no. GFWY00000000). Transcripts with the best matches with the used arthropod genes, in terms of similarity and e-values, were extracted from the transcriptome and used to generate the primers (S2). The two reference genes, *HIST* and *ATPS*, were prior the study selected among 7 different genes to be the most stable across different developmental stages (eggs, adults) and stressors (salinity shock, handling - and density stress) after assessment with the geNorm method in the NormqPCR R package [52].

To ensure the identity of the primer product sequences originated from the *A. tonsa* transcriptome, conventional PCR were used to generate PCR products and submitted for sequencing at Eurofins Genomics (Ebersberg, Germany).

The Brilliant® II Master Mix (Sigma-Aldrich, USA) kit was used for setting up qPCR according to manufactures protocol using 2 µL cDNA as template. The reactions were run on Stratagene Mx3005P (AH Diagnostics, Aarhus V, Denmark) thermal cycler as follows: [95°C/15 min]; 40 cycles: [95°C/30s] [58°C/60s] [72°C/30s]. At the end of the cycling program, a melting curve analysis where added. Each replicate sample was run in technical triplicates. PCR amplification of each primer was prior the gene expression analysis determined by the standard curve method, and all had efficiencies above 80%. Gene expression was normalized using the geometric mean of the two reference genes *HIST* and *ATPs* and the $2^{\Delta\Delta\text{-CT}}$ method to estimate relative mRNA levels [53].

Statistics

Since the gene expression levels were heavily skewed on the linear scale, the relative mRNA levels were log2 transformed prior the statistical analysis. Statistical significance of developmental time versus log2 fold change (log2FC) was determined using a one-way ANOVA followed by a Turkey's post-hoc test. All data were tested for normality, homogeneity, and independence prior the ANOVA analysis. All data analysis and statistics were done using R [54].

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SUPPLEMENTARY

S1*. Selected developmental times for sampling embryos of the subitaneous development and quiescent state for real-time quantitative PCR and description of the corresponding developmental stages.*

Time	Subitanous development	Quiescent state
1 h	Majority of embryos in a single- or two celled stage (S1, S2)	Majority of embryos in a single- or two celled stage (S1, S2)
4 h	Majority of embryos in a 64 (S7), 128 – or more celled stage (S8).	S3 – S4
5 h	Majority of embryos beginning gastrulation (G)	Embryos ranging from a 16- to a 64-celled stage (S5, S6, S7).
7 h	Majority of embryos in gastrulation (G)	16-celled stage (7S)
12 h	Beginning organogenesis (O)	128-celled stage – or more (S8)
16 h	Majority in organogenesis (O)	128-celled stage – or more (S8)
32 h	Majority in limb bud stage, LB	128-celled stage – or more (S8)
48 h	Early or final nauplii, EN or FN	Gastrulation (G)
7d		Gastrulation (G)

S2. *Real-time quantitative PCR primers for the following genes: ecdysteroid-phosphate phosphatase (EPPase), ecdysone receptor (EcR), ß fushi tarazu transcription factor 1 (ßFTZ-F1), ecdysteroid-regulated early gene E74 (E74), ATP synthase (ATPS) and Histone 3 (HIST). Accession numbers, similarity percentages and E-values are given for the species used to search for the gene sequences in the Acartia tonsa transcriptome with accession number: GFWY00000000. The sequences were extracted from the transcriptome and the primers generated. F: forward primer, R: reverse primer.*

Gene	Primers	Amplicon	Accession	Similarity	E-value
EPPase	**F:** CATGGAGAGAGGGTAGACTTTACT **R:** AAATCTTTTCTTTCATAGTTTCCAGCA	83 bp	AB742158 *Daphnia magna*	72.8 %	1.69e-05
EcR	**F:** CGGCCACAGGAGGAATTTTG **R:** CGGAAAAATCCCTTGCACCC	95 bp	JX105044 *Bactrocera dorsalis*	68.3%	1.62e-24
ßFTZ-1	**F:** CACTCCGACACAAAGGAAGGA **R:** GACTCGCAGGTTAGCAGGC	92 bp	LC105701 *Daphnia magna*	79.4%	6.19e-19
E74	**F:** GTACCGGGCCTGTATCTGTG **R:** GAGGAAAGGCTCCCCGTAAG	95 bp	NM001043979 *Bombyx mori*	75.1%	2.31e-44
ATPS	**F:** TCCAGAAGGAACGCCGGC **R:** AGCACCTAGACGAACAGCTA	100 bp	NC014887.1 *Acrida cinerea*	69.1%	1.22e-24
HIST	**F:** GTACCGGGCCTGTATCTGTG **R**: GAGGAAAGGCTCCCCGTAAG	100 bp	NW001887649.1 *Culex quinquefasciatus*	79.5%	1.16e-99

Discussion of transcriptional methods

Transcriptional methods estimate the quantity of RNA molecules transcribed from the DNA sequences of genes (i.e., transcription) and thereby provide quantitative measures of gene expression under different experimental treatments. Transcriptional methods include targeted (e.g., real-time quantitative PCR), semi-targeted (e.g., DNA microarray) and transcriptome-wide (e.g., RNA sequencing) approaches. The choice of method depends primarily on the scientific research goal, funding available, and the availability of a reference genome or transcriptome for the organism in question. The focus of my Ph.D. project is on protein-encoding genes, thus the focus of the methods will be on messenger RNA (mRNA). It is, however, important to note that mRNA only accounts for a small fraction (~5%) of RNA molecules present in the cell. The majority of RNAs are ribosomal RNAs (rRNAs), transfer RNAs (tRNAs,) and microRNAs (miRNAs) (Lodish et al., 2000).
The majority of the work described in this dissertation is based on two transcriptional approaches: targeted gene expression analysis using real-time quantitative PCR (real-time qPCR) and transcriptome-wide assessment with RNA sequencing (RNA-Seq). Here, I will discuss the most important considerations regarding the methods used.

Real time quantitative PCR

During real-time quantitative PCR (real-time qPCR), the PCR amplification of targeted complementary DNA (cDNA, reverse transcribed from mRNA) is monitored in real time (i.e., during the course of the PCR amplification reactions). By using a fluorescent dye that is either intercalated with double-stranded DNA or sequence specific probes (e.g., SYBR green or TaqMan), the exponential amplification of the PCR product can be monitored and used to determine the initial quantity of the target DNA (VanGuilder et al., 2008). PCR does in theory amplify DNA exponentially, doubling the number of target molecules for each amplification cycle. In real-time qPCR, the amounts of PCR product are measured after each cycle based on the increasing concentration of the fluorescent label, which is in direct proportion to the number of amplicons generated. By plotting the fluorescence against cycle number during the real-time qPCR reaction, an amplification plot is generated that represents the accumulation of PCR product for each cycle throughout the real-time qPCR reaction (VanGuilder et al., 2008). The changes in mRNA levels of a gene (target gene) can be determined across different conditions relative to the levels of an internal control (i.e. reference gene). If the target sequence is abundant in the initial sample, the labeled amplification product concentration will increase more rapidly and cross a pre-set critical threshold level (Ct) at an earlier PCR cycle than samples with lower concentrations of target PCR product (Livak and Schmittgen, 2001). Thus the gene expression can be estimated as the relative amount of mRNA between the two samples (Livak and Schmittgen, 2001).
Gene expression analysis by real-time qPCR can be done using very low amounts of PCR products and is relatively inexpensive compared to RNA-Seq (VanGuilder et al., 2008). The

method has a wide dynamic range of quantification, is less biased than RNA-Seq, and is suitable for measuring expression for a limited number of genes simultaneously in many different samples (Klein, 2002; VanGuilder et al., 2008). Another advantage of real-time qPCR is that there is an established set of minimal standard requirements (MIQE) for best practices to ensure reliability and repeatability, which is lacking for RNA-Seq (Bustin et al., 2009). One major disadvantage of real-time qPCR is that it requires species-specific primers designed from existing sequence data for the target organism. Since *Acartia tonsa* lacked a reference genome or transcriptome at the time this manuscript was produced, only a few genes with available reference sequences could be included in the analysis. Additional advantages and limitations of real-time qPCR can be found in Table 2.

Table 2. Advantages and limitations of real-time quantitative PCR.

Advantages	Limitations
Wide dynamic range of quantification.	Only target genes analyzed, versus whole-transcriptome from RNA sequencing.
Low cost.	Needs existing sequence data for target genes from the species to be examined..
High sensitivity to detect low abundance transcripts.	Needs validation in comparison with non-responding or reference genes (i.e., housekeeping genes).
Established best practices (MIQE).	Needs primer and PCR protocol optimization.
Less biased than Microarray and RNA sequencing.	Technical replicates needed to control for differences in RNA quality, amount of starting material, pipetting errors etc.
More tolerant of partially-degraded RNA compared to RNA-sequencing.	

The selection and validation of reference genes (i.e., housekeeping genes), as well as biomarkers, is crucial when designing real-time qPCR experiments to answer biological questions. In manuscript III it was shown that handling affects gene expression of potential biomarkers. In this discussion, potential reference genes for use in manuscript II are validated.

Validation of reference genes

Relative changes in mRNA levels can be estimated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method described by Livak and Schmittgen (2001). The method accounts for systematic variation (e.g., differences in starting material, RNA quality, PCR efficiencies etc.) by normalizing expression against one or more reference genes (Chervoneva et al., 2010; Livak and Schmittgen, 2001). It is, however, strongly recommended to normalize the resultant data by calculating the geometric mean of multiple reference genes when possible (Vandesompele et al., 2002). The ideal reference gene displays stable expression across all the analyzed samples, regardless of experimental conditions (Svingen et al., 2015). Unfortunately, many gene expression studies using real-time qPCR are done without proper validation of the reference genes used (Table 3). Commonly-used reference genes are often carry-overs from older studies using semi-quantitative methods, e.g., Northern blots, RNase protection assays, and conventional reverse-transcription PCR assays (Huggett et al., 2005). The reality is that these genes rarely exhibit stable expression across different conditions, thus it is important to determine which genes are suitable as references, with stable expression across different cell types and developmental stages, regardless of experimental conditions (Dheda et al., 2005; Huggett et al., 2005; Kozera and Rapacz, 2013).

The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).
From Ct values of target gene(s) during treatment (TT) and control (TC) conditions, as well as reference gene(s) during treatment (RT) and control (RC) conditions, ΔΔCt can be calculated as:

$$\Delta\Delta Ct = (TT - RT) - (TC - RC)$$

PCR product are being doubled for each cycle, thus the calculations are in log2. The $2^{-\Delta\Delta Ct}$ gives the expression fold-change.

Table 3. Commonly used reference genes in copepods.

Gene	Species	Reference	Validation?
ß-actin (ACT)	*Acartia tonsa*	(Aguilera et al., 2016)	No
		(Nilsson et al., 2017, 2013)	No
		(Petkeviciute et al., 2015)	No
		(Rahlff et al., 2017)	No
	Tigriopus japonicus	(K. W. Lee et al., 2008; Lee et al., 2007)	No
		(Rhee et al., 2009)	No
		(Seo et al., 2006a)	No
		(Lee et al., 2017)	Yes
	Tigriopus californicus	(Chan et al., 2014)	No
	Calanus finmarchicus	(Hansen et al., 2008)	Yes
		(Roncalli et al., 2016b)	Yes
	Calanus helgolandicus	(Lauritano et al., 2016, 2013, 2011b)	Yes
	Calanus sinicus	(Lauritano et al., 2015)	Yes
	Eurytemora affinis	(Rahlff et al., 2017)	No
	Paracyclopina nana	(Hwang et al., 2010b)	No
	Pseudodiaptomus annandalei	(Jeong et al., 2015)	Yes
		(Jiang et al., 2013)	No
	Pseudodiaptomus poplesia	(Zhuang et al., 2017)	Yes
Elongation factor (EFA)	*Acartia tonsa*	(Nilsson et al., 2017, 2013)	No
		(Petkeviciute et al., 2015)	No
	Calanus finmarchicus	(B. H. Hansen et al., 2010; Hansen et al., 2008)	Yes
		(Roncalli et al., 2016b)	Yes
	Calanus helgolandicus	(Lauritano et al., 2016, 2013, 2011b)	Yes
	Calanus sinicus	(Lauritano et al., 2015)	Yes
		(Zhou et al., 2016)	No
	Lepeophtheirus salmonis	(Tribble et al., 2007)	No
		(Borchel et al., 2017)	Yes
	Tigriopus japonicus	(Park et al., 2017)	No
		(Lee et al., 2017)	Yes
	Paracyclopina nana	(Jeong et al., 2015)	Yes
	Pseudodiaptomus poplesia	(Zhuang et al., 2017)	Yes
18S rRNA (18S)	*Tigriopus japonicus*	(Yi et al., 2014)	No
		(B.-M. Kim et al., 2015; Kim et al., 2014, 2011, 2013a, 2013b)	No
		(Lee et al., 2017)	Yes
		(Jeong et al., 2016, 2014)	No
		(Han et al., 2015b)	No
		(Rhee et al., 2013)	No
		(Hwang et al., 2016)	No
	Calanus sinicus	(Lauritano et al., 2015)	Yes
	Calanus helgolandicus	(Lauritano et al., 2013, 2011b)	Yes
	Paracyclopina nana	(Lee et al., 2012, 2016)	No
		(Jeong et al., 2015)	Yes
		(Puthumana et al., 2017)	No
		(Han et al., 2015a)	No
Ribosomal protein S16 (S16)	*Calanus finmarchicus*	(Aruda et al., 2011)	No
		(Roncalli et al., 2016b)	Yes
	Calanus sinicus	(Zhou et al., 2016)	No
Ribosomal protein S20 (S20)	*Calanus helgolandicus*	(Lauritano et al., 2016, 2013, 2011b)	Yes
	Calanus sinicus	(Lauritano et al., 2015)	Yes
	Pseudodiaptomus poplesia	(Zhuang et al., 2017)	Yes
Ribosomal protein S7 (S7)	*Calanus helgolandicus*	(Lauritano et al., 2016, 2013, 2011b)	Yes
	Calanus sinicus	(Lauritano et al., 2015)	Yes

Table 3. *Continued from previous page.*

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	***Tigriopus japonicus***	(Lee et al., 2006, 2017)	**Yes**
		(Seo et al., 2006b, 2006c)	No
	Tigriopus californicus	(Barreto et al., 2015b)	No
	Calanus helgolandicus	(Lauritano et al., 2016, 2013, 2011b)	Yes
	Calanus sinicus	(Lauritano et al., 2015)	Yes
		(Zhou et al., 2016)	No
	Paracyclopina nana	(Jeong et al., 2015)	Yes
	Pseudodiaptomus annandalei	(Jiang et al., 2013)	No
Ubiquitin (UBI)	*Calanus helgolandicus*	(Lauritano et al., 2013, 2011b)	Yes
	Calanus sinicus	(Lauritano et al., 2015)	Yes
Histone H3 (HIST)	*Calanus helgolandicus*	(Lauritano et al., 2016, 2013, 2011b)	Yes
	Calanus sinicus	(Lauritano et al., 2015)	Yes
ATP synthase (ATPS)	*Calanus helgolandicus*	(Lauritano et al., 2013, 2011b)	Yes
	Calanus sinicus	(Lauritano et al., 2015)	Yes

At the time manuscript I was produced sequencing data for *Acartia tonsa* was very limited, and there were very few potential reference genes. Of the two available reference genes, *ß-actin* (*ACT*) and *elongation factor α* (*EFA*) originating from degenerated primer design, only *ACT* exhibited stable expression, suitable for use as a reference gene, for the density conditions and incubation periods used. After generating the *de novo* transcriptome for *Acartia tonsa* in manuscript II, it was possible to generate new reference genes for manuscript III.

Based on commonly-used reference genes in copepods (Table 3), eight candidate genes were selected for validation: *ACT*, *EFA*, *ribosomal protein S20* (*S20*), *Ubiquitin* (*UBI*), *18S rRNA* (*18S*), *histone H3* (*HIST*), *ATP synthase* (*ATPS*) and *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) (Table 3). The validation method (e.g., GeNorm) described in Vandesompele et al. (2002) recommends eight reference genes, but after multiple failed attempts with *GAPDH* (e.g., failed primer design, PCR efficiencies below 80%, etc.) this gene was excluded from the validation. The remaining seven reference genes were validated across the six treatments to represent conditions described in the three manuscripts (Table 4).

The candidate genes were validated across the six treatments given in Table 4 with triplicates.

Table 4. Treatments used for reference-gene validation.

Control	Adult individuals (N=25) of *Acartia tonsa* not exposed to handling or other stressors within their lifespan as described in manuscript II and kept at a density around 100 ind. L^{-1} as described in manuscript I (100 eggs were hatched in 1 L beaker glasses, resulting in ~80-100 nauplii).
Density	Adult individuals (N=25) of *Acartia tonsa* exposed to a high-density condition (10,000 ind. L^{-1}) for 8h as describe d in manuscript I.
Handling	Adult individuals (N=25) of *Acartia tonsa* exposed to 10 min handling on a plankton net screen (250μm) followed by 1.5h incubation at regular culture conditions as described in manuscript II.
Salinity	Adult individuals (N=25) of *Acartia tonsa* exposed to 10 min salinity shock (from S=35 to S=5) followed by 1.5h incubation at regular culture conditions as described in manuscript II.
Sub. 1h	Eggs (N=100) subitaneous developed for 1h as described in manuscript III.
Sub. 32h	Eggs (N=100) subitaneous developed for 32h as described in manuscript III.

The incubation period of 1.5 h for Handling and Salinity were selected based on the assumptions in manuscript III that a stress response will occur between 15 h and 24 h after exposure to the corresponding treatment. According to Petkeviciute et al. (2015), and Rahlff et al. (2017) *heat-shock protein 70kDa* (*HSP70*) exhibits a peak in expression within a few hours after exposure to stress. To address this, expression of *HSP70* was also analyzed across the six treatments and served as a positive control for the stress experiments (Table 5).

RNA extraction, production of cDNA by reverse transcription, and real-time qPCR were performed as described in manuscript III. In addition to the six treatments, cDNA originating from bulk *A. tonsa* was serial-diluted to generate a standard curve for PCR efficiency estimation as described in Pfaffl et al. (2004). Only primers with PCR efficiencies above 80% were used for further analysis (Table 5).

PCR efficiency (E)

The slope of a linear regression model, originating from a dilution series of cDNA concentrations plotted against the Ct-values. The maximal efficiency is E=2 corresponding to that each cycle of PCR results in a doubling in PCR product, hence the efficiency is 100%. The minimal value is E=1, corresponding to no replication of PCR product.

$$\mathbf{E = 10^{-1/slope}}$$

Table 5. Real-time quantitative PCR primers for *ribosomal protein S20* (*S20*), *Ubiquitin* (*UBI*), *18S rRNA* (*18S*), *histone H3* (*HIST*), *ATP synthase* (*ATPS*), *ß-actin* (*ACT*), *elongation factor α* (*EFA*) and *heat-shock protein 70kDa* (*HSP70*). Accession numbers are given for the species used to search for the gene sequences in the *Acartia tonsa* transcriptome (GFWY00000000.1) generated in manuscript II. The sequences were extracted from the transcriptome and primers generated in Geneious (ver. 9.1.6). For *ACT* and *EFA* the corresponding references are given. Standard curve R^2 range: 0.97-0.99. F: forward primer, R: reverse primer.

Gene	Primers	Size (bp)	Accession/reference	PCR efficiencies
S20	**F**: GGAGGCGTGCAGATCAATGA **R**: CCACCCGTAAGACACCTTGC	92	NW004581745.1	93.77%
UBI	**F**: CAAGACCATCACCCTGGAGG **R:** CAATCTCTGCTGGTCTGGGG	100	NW011627326.1	96.29%
18S	**F**: GTCGCCTATCACCACGTCAA **R**: GAGACTGACGGGGGCATATG	213	FJ422281	99.35%
HIST	**F:** GTACCGGGCCTGTATCTGTG **R**: GAGGAAAGGCTCCCCGTAAG	100	NW001887649.1	98.90%
ATPS	**F:** TCCAGAAGGAACGCCGGC **R:** AGCACCTAGACGAACAGCTA	100	NC014887.1	98.16%
ACT	**F**: CTTCTGCATACGGTCAGCAA **R:** ACCCGTACGCCAACACTG	54	(Nilsson et al., 2013)	91.49%
EFA	**F**: TCTTAAGCCCGGTATGATCG **R**: GAGACTCGTGGTGCATCTCA	42	(Petkeviciute et al., 2015)	83.97%
HSP70	**F:** TTCAATGATTCACAGAGACAAGC **R:** TCCTTGTGATGTTAAGACCAGCTAT	200	(Nilsson et al., 2013)	97.99%

Expression patterns for reference genes should be similar, therefore the threshold cycles (Ct) were screened for the candidate genes (Fig. 2A, Kozera and Rapacz, 2013). The high expression of *18S rRNA* (Fig. 2A), corresponding to lower Ct values, makes it unacceptable as a reference gene for *A. tonsa* under the six experimental conditions. The same high *18S* expression has been observed in another copepod, *Calanus sinicus*, during exposure to toxic diatoms (Lauritano et al., 2015). This may suggest that *18S* is not suitable as a reference gene for copepods in general.

In order to identify the most suitable reference genes among the candidates, I initially used the geNorm algorithm (Perkins et al., 2012; Vandesompele et al., 2002). After finalizing the study described in manuscript II, I became aware of the additional algorithms BestKeeper (Pfaffl et al., 2004) and NormFinder (Andersen et al., 2004).

For future gene expression studies with *A. tonsa*, I (re)analyzed the seven candidates using the three algorithms.

BestKeeper assumes that reference genes have similar expression patterns; hence suitable reference genes should have highly-correlated expression patterns. From the geometric mean of Ct values, standard deviation (SD) and a BestKeeper index are estimated. Genes that are stably expressed have a SD below 1. The genes are compared pairwise, and those with the lowest SD values (<1) and coefficients of correlation (r) closest to 1 compared to the BestKeeper index are assumed to exhibit the most stable expression among the candidate genes (Pfaffl et al., 2004). From the BestKeeper results (Table 6, Fig. 2B), *HIST* is the

gene with the lowest SD and highest r, followed by *18S*, which was found to be inappropriate as a reference because of its highly variable expression (Table 6, Fig. 2A).
NormFinder uses a statistical linear mixed-effect model to estimate intra- and inter-group variation of gene expression, and combines the two into a stability value. The genes with the lowest stability value are assumed to be the most stable across the experimental conditions (Andersen et al., 2004). From the NormFinder analysis *UBI* and *S20* were found to be the most stable (Fig. 2C, Table 6).
geNorm (Perkins et al., 2012; Vandesompele et al., 2002) calculates a gene stability value (M), which is defined as the average pairwise variation of gene expression. The procedure is iterative, where the least-desirable reference gene is discarded, with subsequent recalculation of the M-values. The ranking of M-values is carried out in a step-wise manner starting with the two genes having the lowest pairwise variation, which in this analysis was *HIST/ATPS* (Fig. 2D, Table 6, Vandesompele et al., 2002). M-values lower than 1.5 are recommended for selecting stable reference genes, which was the case for all the candidate genes tested (Fig. 2D, Vandesompele et al., 2002).

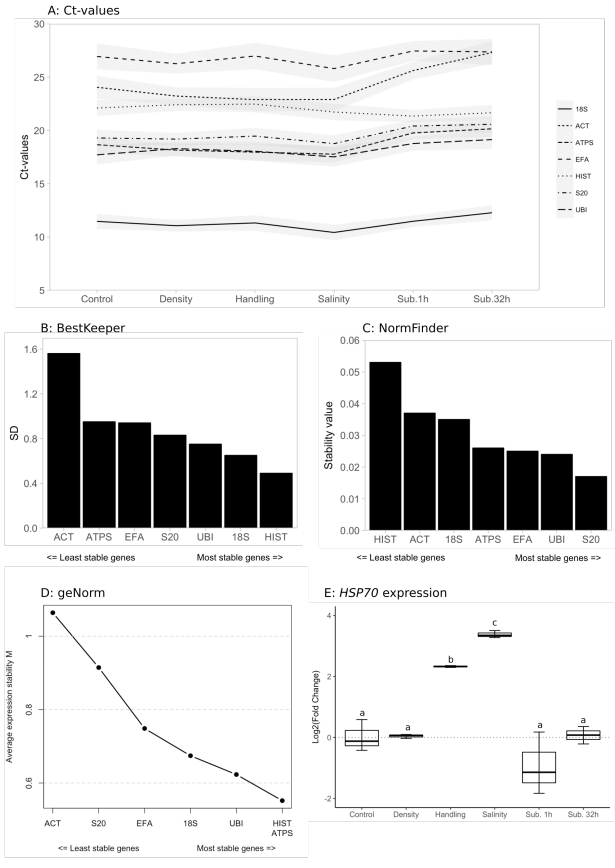


Figure 2. A) The threshold cycle (Ct) values for potential reference genes in the copepod *Acartia tonsa* during a Control (no-handling or stress applied), Density (10,000 ind. L^{-1} for 8h), Handling (10 min on a 250µm plankton net followed by 1.5h at regular culture conditions), Salinity (10 min salinity shock from 35 to 5 salinity units, followed by 1.5h at regular culture conditions), Sub. 1 h (subitane-

Figure 2. *Continued from previous page.*
ous development for 1h), and Sub. 32 h (subitaneous development for 32h). Each curve represents the degree of stability of Ct values for each reference gene. The shadow around each curve represents the 95% confidence limit. **B)** Assessment of potential reference genes across the conditions described in A) for the copepod *Acartia tonsa*, by rankings obtained with BestKeeper. The most suitable reference genes have the lowest Ct standard deviation. **C)** Potential reference gene assessment ranked by the lowest stability value calculated with NormFinder as the most stable genes. **D)** Potential reference gene assessment ranked by the lowest average expression stability (M) estimated using geNorm as the most stable genes. E) Heat-shock protein 70kDa *(HSP70*) expression in *Acartia tonsa* for: Control, Density, Handling, Salinity, Sub.1h, and Sub. 32h (Table 4). Expression levels were normalized against the geometric mean of the reference genes, *HIST* and *ATPS*. Error bars represent standard deviation. Tukey HSD was used to test for significant differences in gene expression (log2 Fold-Change) between treatments. Different letters indicate statistically significant differences ($P < 0.05$).

Unexpected changes in reference gene expression can cause erroneous results and thereby wrong conclusions about real biological effects. Therefore it is crucial to validate potential reference genes before using them for normalization in gene expression studies. The geometric mean of *HIST* and *ATPS* (suggested by the initial assessment with geNorm) was used for normalization of gene expression in manuscript III based on the findings with geNorm. The three approaches (e.g., BestKeeper, NormFinder and geNorm) gave different results depending on software type. The expression of *HIST* and *ATPS* were, however, stable between the samples representing subitaneous development and quiescence (Fig. 3), hence the selection of these genes was considered reliable for manuscript III.

Arithmetic and geometric mean.
From 3 hypothetical genes, A, B, and C, the arithmetic and geometric mean of gene expression can be calculated as:

Arithmetic mean: (A + B + C) / 3

Geometric mean: $(A * B * C)^{1/3}$

Table 6. Ranking of the best reference genes as given by analysis with BestKeeper, NormFinder and geNorm**.** SD: standard deviation, Ct: threshold cycle, r: Pearson correlation coefficient of the Bestkeeper index versus the gene. All Pearson correlation coefficients had p-values < 0.001.

Ranking	BestKeeper	SD±Ct	r	NormFinder	Stability value	geNorm	Average stability M
1	HIST	0.48	0.98	S20	0.017	HIST	0.551
2	18S	0.64	0.90	UBI	0.024	ATPS	0.551
3	UBI	0.75	0.85	EFA	0.025	UBI	0.623
4	S20	0.82	0.95	ATPS	0.026	18S	0.674
5	EFA	0.94	0.81	18S	0.035	EFA	0.748
6	ATPS	0.95	0.89	ACT	0.037	S20	0.914
7	ACT	1.56	0.90	HIST	0.053	ACT	1.064

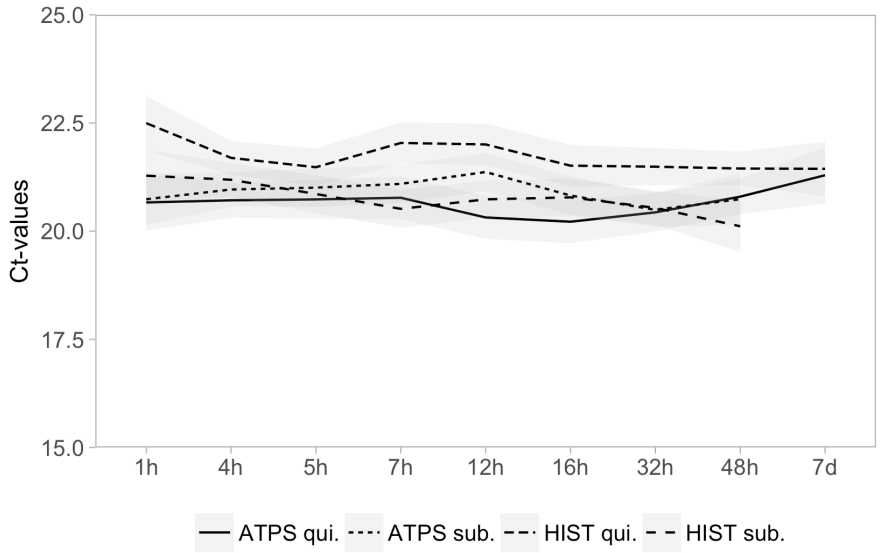


Figure 3. The threshold cycle (Ct) values for *ATPS* and *HIST* over time during subitaneous development and quiescence of *Acartia tonsa*, described in manuscript III. Each curve represents the degree of stability of Ct values for the two selected reference genes. The shadow around each curve represents the 95% confidence limit.

In addition to examining gene stability with multiple algorithms, I also became aware of the function of geNorm for finding the optimal number of reference genes for normalization by calculating the pairwise variation between sequential normalization factors (Perkins et al., 2012; Vandesompele et al., 2002). The cutoff value of 0.15 is considered as the ratio above which gene expression analysis will not benefit from addition of reference genes for normalization (Perkins et al., 2012; Vandesompele et al., 2002). Across all the tested conditions, four reference genes are recommended for future studies (Fig. 4). Adding an additional fifth reference gene would not increase the ratio more than 0.15.

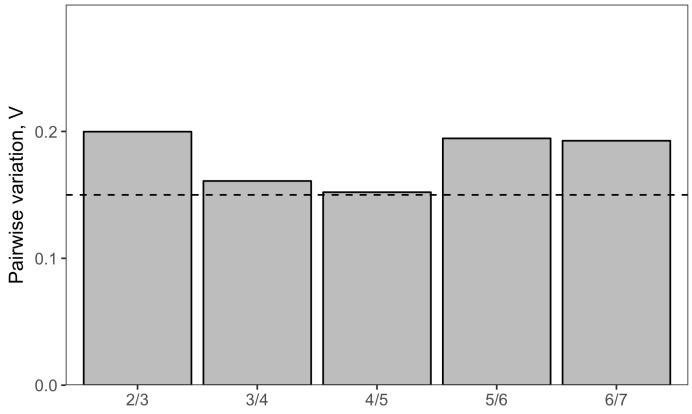


Figure 4. geNorm pairwise variation (V) analysis for finding the optimal number of reference genes for normalization of gene expression analysis in *Acartia tonsa*.

The *HSP70* expression served as a positive stress control to validate the results of both reference genes, as well as the findings in manuscript I and II (Fig. 2E).
Handling stress resulted in a 5.2±0.3 (mean±SD) fold up-regulation of HSP70 (log2FC=2.4, Fig. 2E) and the salinity shock in a 13.3±0.8 fold up-regulation (log2FC=3.7, Fig. 2E). The non-significant expression of *HSP70* for the Density treatment in relation to the Control were consistent with the findings of manuscript I (Fig. 2E). The subitaneous development *of A. tonsa* after 1h and 32 h did not exhibit any significant changes in *HSP70* expression (Fig. 2E). The expression of *HSP70* measured 1.5 h following exposure to Handling and Salinity confirms that a peak in stress response occurs between 15 min and 24 h following exposure. Description of the treatments can be found in Table 4.

RNA sequencing

The RNA sequence reflects the DNA sequence from which it was transcribed. The transcriptome of an organism reflects the sum of all messenger RNA (mRNA) transcribed from genes at a specific time. Analyzing the transcriptome in relation to different experimental treatments provides an overview of which genes are expressed and therefore may be hypothesized to play a role in response to those conditions (Marguerat and Bähler, 2010).
RNA sequencing (RNA-Seq) uses Next Generation Sequencing (NGS) technologies to reveal the presence and quantity of mRNA molecular present at different conditions. In contrast to real-time quantitative PCR (real-time qPCR), this method is untargeted, and measures gene expression across the entire transcriptome, which makes possible novel discoveries of genes, DNA variations and alternative splice variants (Wang et al., 2009). The major advantage of RNA-Seq is the high level of information that can be provided from a single data set. But RNA-Seq is expensive and requires additional considerations in experimental planning, including consideration of sequencing technology, type of library preparation, sequencing depth, sample size and number of replicates. In addition, guidelines for best practices for bioinformatics are limited, since sequencing technologies advance rapidly and available bioinformatics pipelines become quickly outdated, slow or fail to work (Mykles et al., 2016). Advantages and limitations of RNA-Seq can be found in Table 7.

Table 7. Advantages and limitations of RNA sequencing

Advantages	Limitations
High level of information.	Expensive.
Whole-transcriptome analysis of gene expression.	Lack of guidelines for best practices for both sequencing and bioinformatics.
Doesn't require available sequence data.	Additional considerations regarding experimental design.
No need for technical replications .	Bioinformatic analysis requires large computing capacity.
Can detect and analyze novel genes, alternative splice, and rare allelic variants.	

Sufficient sequencing depth (i.e., number of reads per sample) is needed to ensure that the whole transcriptome of an organism is properly covered by RNA-Seq (i.e., sequencing coverage; Wang et al., 2009). Greater coverage requires deeper sequencing depth, which can be costly for research budgets. Furthermore, replicates are most likely necessary in order to maximize statistical power to detect differential gene expression. The number of biological replicates should be sufficient to achieve maximum statistical power (within any budget constraints), but also allow sufficient sequencing depth to detect weakly expressed genes.

During *de novo* transcriptome assembly, sequence polymorphism will increase the complexity of the *de bruijn* graph (Iqbal et al., 2012; Paszkiewicz and Studholme, 2010). Since this will have a potentially negative effect on the assembly, the reference transcriptome should be generated from a sample as homogeneous as possible (MacManes, 2015). Since multiple individuals would result in higher levels of polymorphism, a single individual of *Acartia tonsa* was sequenced at a depth of ~350 million reads. This also prevented shallow coverage due to dilution of sequencing depth across multiple individuals. The goal for the reference *de novo* transcriptome described in manuscript II was to ensure sufficient sequencing depth to allow weakly-expressed genes to be represented in the assembly. Comparison with transcriptome assemblies of other copepod species can be found in Table 8. In general, sequencing depths of >100 million reads have been recommended for *de novo* transcriptome assemblies (Liu et al., 2014; Myers et al., 2011).

Table 8. Copepod transcriptome assemblies. N50 stastistics were performed in 2017 using publicly accessible NCBI transcriptome shortgun assemblies (TSA). For studies without available TSA, the Sequence Read Archive (SRA – marked with SRP in accession no.) or reference are given. Platform gives the used sequencing platform. type is the type of sequencing in terms of read length (bp) and if it is paired-end (PE), or single-end (SE) sequencing. #Rep gives the number of samples used for the transcriptome assembly. Depth is the sequencing depth in Million (M) reads. Software gives the used assembly software (CLC: CLC Genomics Workbench). #Contigs is the number of resulting contigs after assembly. The N50 value, is when at least 50% of the assembled bases are in contigs of at least the N50 value length in bp. Species: *Acartia tonsa* (*A. tonsa*); *Calanus finmarchicus (C. finmarchicus); Calanus glacialis (C. glacialis); Calanus sinicus (C. sinicus); Eurytemora affinis (E. affinis), Labidocera madurae (L. madurae); Neocalanus flemingeri (N. flemingeri); Paracyclopina nana (P. nana); Pseudocalanus acuspes (P. acuspes); Tigriopus californicus (T. californicus); Tigriopus japonicus (T. japonicus); Tigriopus kingsejongensis (T. kingsejongensis).*

* cleaned sequences
** Numbers given in reference since there were no available TSA for reproducing the results.for the transcriptomes

Species	Sample	Platform	Type	#Rep	Depth	Software	#Contigs	N50	Resources
A. tonsa	1 adult female	Illumina NextSeq	150 PE	1	~350M	Trinity	60,662	1,874	Manuscript II GFWY00000000
	4 eggs 5 nauplii, copepodites 6 adults	Illumina Next/Mi -Seq	150 PE	8		Trinity	119,439	1,052	Jørgensen T.S., 2017. HAGX00000000 (AMPHICOP)
C. finmarchicus	400 eggs 180 nauplii 50 late nauplii 40 early copepodites 10 CV stage copepodites 6 adult females	Illumina HiSeq	100 PE	23	~28M/ sample ~640M	Trinity	206,012	1,418	(Lenz et al., 2014). GAXK00000000
	3 CV stage copepodites	Illumina HiSeq	100 PE	16	~93M	Trinity	241,140	988	(Tarrant et al., 2014). GBFB00000000
	5 individuals exposed to short – and long-term thermal stress (3 temperatures)	Ion-Torrent		6	~5M	Trinity	28,954	353	(Smolina et al., 2015). GBXU00000000
C. glacialis	10 nauplii	Illumina HiSeq	50 SE	23	~640M in total	CLC	59,353**	1,019 **	(Bailey et al., 2017a). SRP092884
	10 CV stage copepodites	454 GS FLX		6	~60 – 180K/ sample ~720K in total	Mira	54,344	621	(Ramos et al., 2015). HACJ00000000
	5 individuals short – and long-term stress exposed to 3 temperatures.	Ion-Torrent		6	~3.5M	Trinity	36,880	471	(Smolina et al., 2015). GBXT00000000

Table 8. *Continued from previous page.*

Species	Sample	Platform	Type	#Rep	Depth	Software	#Contigs	N50	Resources
C. sinicus	50 individuals	Illumina HiSeq	100 PE	1	~60M	Trinity	69,751**	1,127 **	(Yang et al., 2014). SRP032493
E. affinis	Females exposed to two strains of Vibrio and females not exposed	Illumina HiSeq	100 PE	1	~25M/ sample ~300M in total	Trinity	138,088	2,089	(Almada and Tarrant, 2016). GBGO00000000
	400 adult females 400 adult males 500 mixed individuals	454 pyroseq.		9	~11 – 50 M/sample 200 M in total	Newbler	19,721**	NA	(Legrand et al., 2016). No TSA or SRA
L. madurae	5-6 adult females 15-26 CIII-CV stage copepodites	Illumina NextSeq	150 PE	3	~530M	Trinity	211,002	1,184	(Roncalli et al., 2017a). GFWO00000000
N. flemingeri	1 adult female	Illumina NextSeq	150 PE	8	~15-20M/sample ~150M in total*	Trinity & CAP3	140,841	1,452	(Roncalli et al., 2017b). GFUD00000000
P. nana	Unknown - adults	Illumina HiSeq	100 PE	1	~200 M	Trinity	60,687	2,148	(Lee et al., 2015) GCJT00000000
P. acuspes	28-74 adults	Illumina NextSeq	50 PE	14	~12 – 25 M/sample ~250 M in total	Trinity	69,555**	851 **	(De Wit et al., 2016). SRP063962
T. californicus	Individuals exposed to two hyperosmotic treatments + non-exposed (bulk)	454 pyroseq.		2	~1M		22,262/ 23,580 **		(Barreto et al., 2011). No TSA or SRA
	4-500 individuals from two distinct populations and two interpopulation crosses	Illumina HiSeq 2000	100 PE	9	~128M and ~49M	CLC	33,710 and 43,077		(Barreto et al., 2015a). SRP049247
T. japonicus	NA	Illumina HiSeq	100 PE	1	~108M	Trinity	54,758	2,211	(H. S. Kim et al., 2015). GCHA00000000
T. kingsejongensis	200 adults	Illumina HiSeq	150 PE	1	~140 M	Trinity	38,250	1,284	(Kim et al., 2016). GDFW00000000

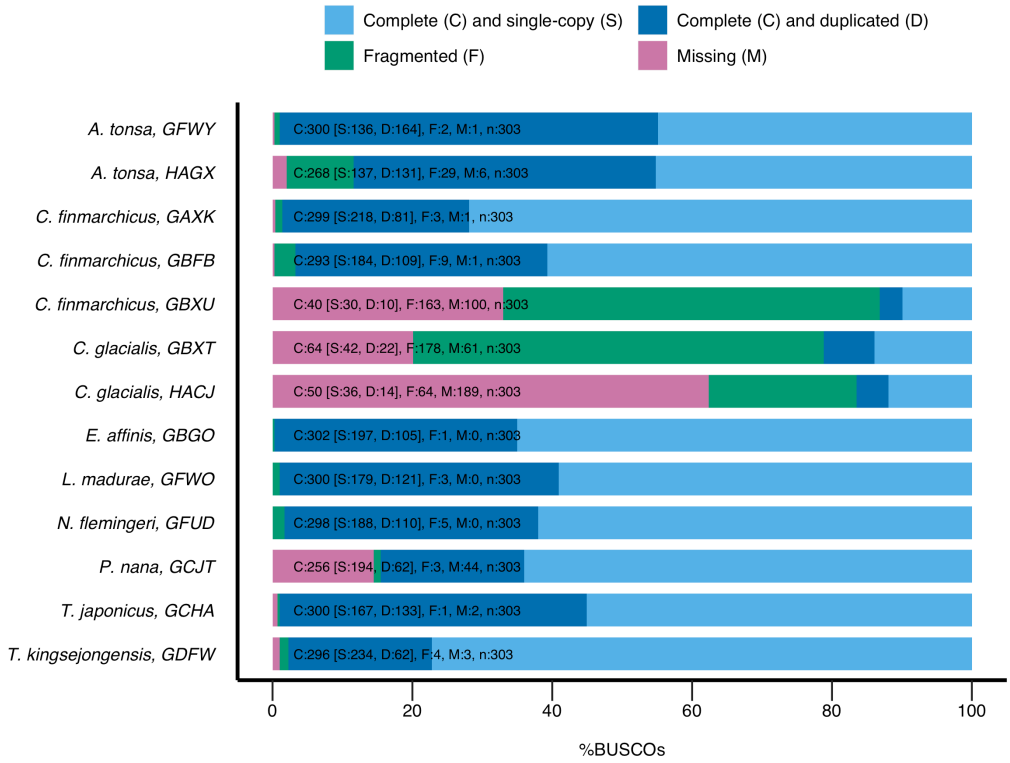


Figure 5. BUSCO analysis of the copepod transcriptomes given in Table 8.

For the results described in manuscript II, experimental conditions entailed triplicates of 10 individuals, which were sequenced at a depth of ~25 million reads per sample in order to determine differential expression, which is consistent with other copepod RNA-Seq studies (Table 8). How the assemblies compare in relation to each other in terms of completeness (i.e. BUSCO analysis, as described in manuscript II) can be found in figure 5.
For differential expression profiling, sequencing depth of 10-30 million reads have been recommended (Francis et al., 2013; Liu et al., 2014; Myers et al., 2011). The pools of 10 adult individuals surely yielded highly diversity (i.e., polymorphism) than a single individual, but pooling has the advantage of minimizing impacts of individual differences in analysis of effects of the treatments. For instance if one of the copepods is better acclimatized in relation to the stressor than others, this will be leveled out by pooling multiple copepods. Very high levels of variation can also make it difficult to properly align the sequences, especially for non-conserved genes, in the *de novo* transcriptome.

The decision about the number of replicates for an experiment is driven by both extrinsic and intrinsic factors. Extrinsic factors include cost, availability of samples, and feasibility of the experiments. Intrinsic factors are knowledge about the biological system, including the stochastic nature of genes that cause variability between samples, genes of special interest and the importance of genes with low levels of expression (Kærn et al., 2005; Liu et al., 2014). Unless the goal is comparing technical aspects of RNA-Seq, usual recommendations are to choose biological, rather than technical, replicates (Liu et al., 2014). The number of

biological replicates should be sufficient to achieve maximum statistical power (within any budget constraints), while still ensuring the sequencing depth is sufficient to detect genes that are weakly expressed. In field studies, the biological variance is typically much higher than for copepods in experimental laboratory cultures, which demands a higher number of replicates in order to achieve the same level of statistical power (Todd et al., 2016). Since the *A. tonsa* strain (identity code: DFH.AT1, Støttrup et al., 1986) used in all three manuscripts has been cultivated since 1981, it is considered to be "biological stable" in comparison to field-caught copepods. The choice of triplicates per treatment for the study described in manuscript II is within the range of numbers of replicates used in other copepod RNA-Seq experiments, of which the majority used field-caught copepods (Table 9).

Table 9. Overview of sequencing depth and number of replicates for differential gene expression assessment in copepods by RNA sequencing. M: million reads. # rep: replicates used per treatment. Type: type of used animals, field caught (including fish-farm caught for sea lice) or culture reared. Seq. depth: sequencing depth.

Species	Treatments	# Individuals	Seq. depth	# rep.	Type	Reference
Acartia tonsa	Control, salinity shock, handling stress	10 adults	~25 M	3	Culture	Manuscript II
Calanus glacialis	Four pH treatments	10 nauplii	~28 M	5-6	Field	(Bailey et al., 2017b)
Calanus finmarchicus	Control, low-dose – and high-dose of dinoflagellate	10 adults	~26 M	3	Field	(Roncalli et al., 2016a)
Eurytemora affinis	Control, exposure to acetone, pyriproxyfen and chlordecone	4-500 adults	~11 – 50 M	3	Field	(Legrand et al., 2016)
Eurytemora affinis	Control, exposure to *Vibrio sp.* (F10) and *V. ordali*	20 females	~25 M	4	Culture	(Almada and Tarrant, 2016)
Tigriopus californicus	Control, two hyperosmotic treatments	Bulk	~0.33M per sample (~1M in total)	1	Field	(Barreto et al., 2011)
Tigriopus californicus	2 distinct populations and 2 interpopulation crosses	4-500 pooled	~3-40 M reads per sample	2-3	Field	(Barreto et al., 2015a)
Pseudocalanus acuspes	Control, 6 pCO_2 exposures, two populations	28-74 adults	~12-25M	1	Field	(De Wit et al., 2016)
Tigriopus californicus	Control (parent populations) and F4 crossed population exposed to lethal – and ambient heat.	30 adults	Unknown	3	Field/Culture	(Kelly et al., 2017)

Table 9. *Continued from previous page.*

Tigriopus californicus	Two populations, 4 temperature treatments	100 pooled	~7.6-29M per sample	1	Field	(Lima and Willett, 2017)
Calanus glacialis	Control and 5 temperature treatments	10 CV stage copepodites	~60 – 180 K per sample	1	Field	(Ramos et al., 2015)
Calanus finmarchicus	Short – and long-term stress exposed to 3 temperatures	5 individuals	~0.8M	1	Field	(Smolina et al., 2015)
Calanus glacialis	Short – and long-term stress exposed to 3 temperatures	5 individuals	~0.6M	1	Field	(Smolina et al., 2015)
Calanus finmarchicus	CIV to CV molt (collected on day 3 and 10)	3 CV stage copepodites	~12M	4	Culture/Field	(Tarrant et al., 2014)

RNA quality is crucial for successful RNA-Seq. For high quality RNA, the RNA integrity number (RIN) should be above 7 and exhibit two clear peaks in a RNA profile (e.g., 28S and 18S rRNA). I experienced issues with very degraded RNA samples when analyzing samples transported to the University of Connecticut (UConn), based on RNA profiles and RIN values measured on an Agilent 2200 TapeStation System (Agilent Technologies, Santa Clara, CA, USA). After multiple failed RNA extractions, I suspected that it either could be degradation during the transport of samples in RNlater to the U.S. or the DNase treatment that caused the trouble. Therefore I extracted RNA from duplicate samples containing 25 "fresh" individuals of *A. tonsa*, stored for 1 week in RNlater (Fig. 6, A1, B1), and duplicates of 10 individuals transported from Roskilde University (Denmark) to University of Connecticut (U.S.A) in RNlater (Fig. 6, C1, D1). For A1, B1, C1 and D1, the RNA extraction was performed without DNase treatment to remove genomic DNA. Furthermore, duplicates of 10 "fresh" individuals stored for 1 week in RNlater (Fig. 6, E1, F1) and 10 individuals transported from Roskilde University (Denmark) to University of Connecticut (U.S.A) in RNlater (Fig. 6, G1, H1) treated with Turbo DNase treatment (Thermo Fisher Scientific) were analyzed.

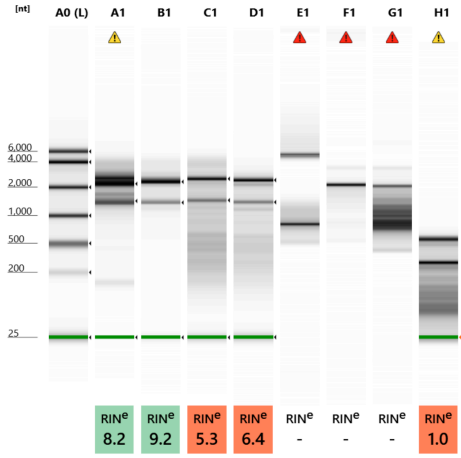


Figure 6. *Acartia tonsa* RNA quality measured on Agilent Tapestation 2200 with RNA High Sensitivity Assay (Agilent Technologies, CA, USA). All samples were extracted with the Qiagen RNeasy minikit. [nt]: RNA size (nucleotides, nt). RIN^e: RNA integrity number equivalent, a RIN^e above 8 is preferable. A0(L): Tapestation ladder. A1 and B1: RNA from 25 (A1) and 10 (B1) individuals stored for 1 week in RNAlater before RNA extraction. No DNase treatment. C1 and D1: RNA from 10 individuals harvested at RUC (Sep. 9, 2016), stored in RNA later at -20°C, transported to UConn in dry ice and then stored at -20°C until RNA extraction on Dec. 8, 2016. No DNase treatment. E1 (N=25) and F1 (N=10): Same as A1 and B1, except with DNAase treatment using TURBO DNA-free™ Kit (Thermo Fisher Scientific, MA, USA). G1 and H1: Same as C1 and D1 with DNase treatment as described in E1 and F1.

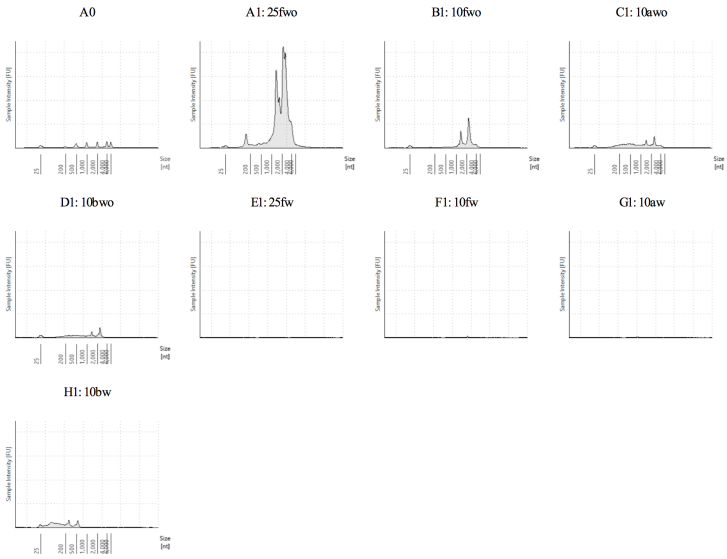


Figure 7. RNA profiles for the samples described in Fig. 6. The overlapping peaks in 28S and 18S rRNA reflects the hidden break (see text for explanation).

RNA extraction from the "fresh" individuals had perfect integrity and quality, as well as a very high concentration (67,200 μg/μL – therefore the alert mark on Fig. 6, A1 for the 25 ind.). Samples stored for longer in RNlater and transported in dry ice from Denmark to the U.S. had a bit more degraded profiles and RINs close to 7 (Fig. 6). The samples furthermore exhibited RNA profiles with signs on the "hidden break" also observed in other arthropods (Fig. 7, McCarthy et al., 2015). The "hidden break" occurs when the RNA is denatured (e.g., heating prior to the Tapestation analysis) resulting in the 28S rRNA break, and generating an odd RNA profile that apprears to merge the 28s and 18s rRNA peaks (Fig. 7). Based on these findings, the DNase treatment was omitted from the protocol.
Since the RINs of the samples were ~7, the Illumina Stranded mRNA Library Preparation Kit (Illumina, Inc., San Diego, CA, USA) was selected for library preparation, which is targeted for protein-encoding mRNAs. As described in manuscript II, the *de novo* transcriptome sample was sequenced in 4 lanes on a NextSeq500 platform, using a mid-output 300 cycle kit (FC-404-2003, Illumina, Inc., San Diego, CA, USA), with 150 bp paired-end reads. The experimental samples were multiplexed and sequenced in two runs across four lanes on the NextSeq500 platform (Illumina, San Diego, CA, USA), with a mid-output 150 cycle kit (FC-404-2001, Illumina, Inc., San Diego, CA, USA) with 75 bp paired-end reads.
Paired-end sequencing allows the sequencing of both ends of a fragment, and thereby generate high-quality sequences that align to the reference transcriptome better than single-end reads (Garber et al., 2011). The paired-end sequencing involves a slightly more complicated library preparation than for single-end sequencing, but yields better final sequencing data (Garber et al., 2011).

Bioinformatics

Despite the lack of best practices for RNA sequencing (RNA-Seq) and subsequent bioinformatics, the number of studies examining transcriptomic responses in crustaceans is rapidly growing (da Fonseca et al., 2016; Mykles et al., 2016). Analysis of RNA-Seq data can be performed both with and without a reference genome. In non-model organisms, such as *Acartia tonsa,* where no reference genome is available, the assembly of a *de novo* transcriptome prior to analysis of differential gene expression is an option.

Since there are no available best practices, the choice of bioinformatics applied in manuscript II will here be discussed. An overview of the applied bioinformatics tool can be found in Fig. 8.

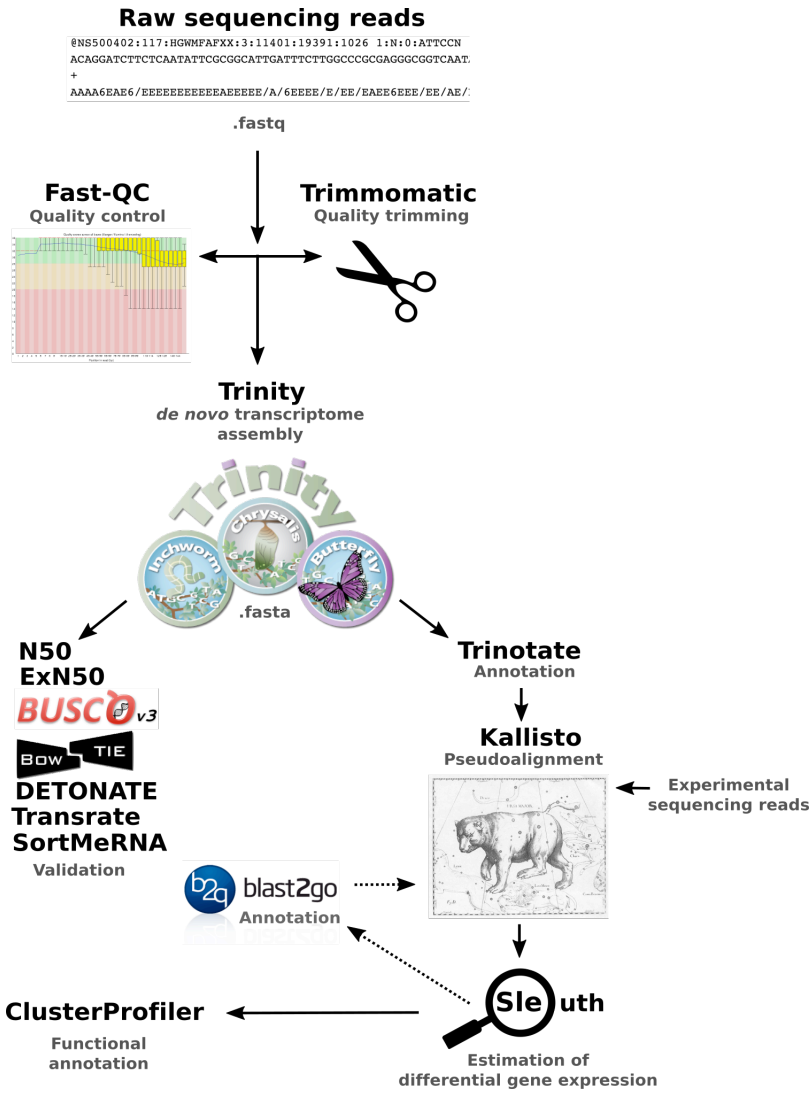


Figure 8. Overview of the bioinformatics tools used in manuscript II. Adapter sequences and low quality reads were removed using Trimmomatic (ver. 3; Bolger et al., 2014). The quality of the reads was checked using FastQC (ver. 0.7, Andrews, 2010). When the quality was accepted, the reads were assembled into a *de novo* transcriptome using Trinity (ver. 2.3.2; Grabherr et al., 2011). Assembly quality was examined by analysis with BUSCO (ver. 2; Simão et al., 2015), realignment of raw reads with Bowtie2 (ver. 2.2.6; Langmead and Salzberg, 2012), N50 and ExN50 statistics (Trinity, ver. 2.3.2; Grabherr et al., 2011), DETONATE (ver. 1.11, Li et al., 2014), Transrate (ver. 1.0.3, Smith-Unna et al., 2016), and SortMeRNA (ver. 2.1, Kopylova et al., 2012). The *de novo* transcriptome was annotated using Trinotate (ver. 3.0.2; Haas et al., 2013) prior differential transcript expression determination with Kallisto (ver. 0.43.0; Bray et al., 2016) and Sleuth (ver. 0.29; Pimentel et al., 2017). Differentially expressed transcripts not annotated by Trinotate were added into Blast2GO (Gotz et al., 2008) to annotate more of the transcripts. Kallisto and Sleuth were then used to determine differentially expressed genes. The gene ontologies assigned to the transcriptome by Trinotate were used for a gene enrichment analysis of the differentially expressed genes using clusterProfiler (ver. 3.6.0, Yu et al., 2012).

Quality control (QC) and filtering

Sequencing reads containing errors tend to contain less biological information, and also negatively affect subsequent alignment and downstream processing (Martin and Wang, 2011). Especially *de novo* transcriptome assemblies require reads of higher quality, in comparison to reads for experimental samples that are aligned to a reference transcriptome in order to determine differential gene expression (MacManes, 2014).

Trimming and filtering of the reads, however, should be done with caution since aggressive removal of reads affects downstream analysis. For instance, very stringent trimming can lead to a decrease in detection of open reading frames (ORF) (MacManes, 2014). Furthermore, this can negatively affect alignment of experimental sample reads to the *de novo* transcriptome and thereby have a negative impact on detection of differential gene expression (Williams et al., 2016).

Gentle trimming can be beneficial for RNA-Seq studies, genome assemblies and Single-nucleotide polymorphism (SNP) identification (MacManes, 2014). Best practices for trimming are an on-going debate (e.g., MacManes, 2014; Williams et al., 2016). Del Fabbro et al. (2013) found best results from trimming at intermediate quality thresholds (Q between 20 and 30). MacManes (2014) suggests that gentle trimming with a Phred score of <2 or <5 is the optimal for most studies.

Not all datasets are similar, therefore best practices are difficult to establish. The best way to find the best trimming options for a data set are a systematic "trial-and-error" approach. Trimming one parameter at a time, with subsequent quality validation, gives a clear overview of what works and what doesn't.

Quality trimming of the data in manuscript II was done using the tool Trimmomatic (Fig. 8, ver. 3; Bolger et al., 2014). Trimmomatic is a tool used for removal of adapter sequences, and trimming and cropping of low-quality reads (Bolger et al., 2014). The quality filtering functionality has a sliding window that scans through the reads from the 5' end and removes bases from the 3' end when the quality within the window drops below a user-specified Phred score (Bolger et al., 2014). After adapter removal, the paired-end mode of Trimmomatic was used with a sliding window across an average of 4 bases to remove low-quality reads with Phred scores (Q) below 10, 20 and 30 (Table 10).

Quality scores were examined between each trimming using FastQC (Fig. 8, Galaxy ver. 0.67, Andrews, 2010). The trimming with Q > 30 were too aggressive and Q >10 contained too many errors, therefore the optimal threshold of Q > 20 was chosen (Table 10). During the library preparation before Illumina sequencing, random hexamers were used as primers for double-stranded cDNA synthesis. This can lead to bias in nucleotide composition in the beginning of reads (K. D. Hansen et al., 2010). Bias was observed in the first 12 bases of the raw reads for the reference transcriptome and was removed by Trimmomatic (Table 10). Reads of >25 and >50 bp were retained for *de novo* transcriptome assembly with Trinity (ver. 2.3.2; Grabherr et al., 2011; Table 10).

Table 10: Sequence trimming with Trimmomatic. # reads: million (M) sequencing reads; Length (bp): length of the reads given in numbers of base pairs; % GC: percentage of Guanine and cytosine; % removal: percentage of reads removal in relation to the raw data. Adap. trim.: removal of sequencing adapters.

	# Reads	Length (bp)	% GC	% Removal
Raw data	354 M	35-151	44	0
Adap. trim.	324 M	35-151	44	8.5
Adap. trim., Q > 10	323 M	26-151	44	8.6
Adap. trim., Q > 20	315 M	2-151	44	11.0
Adap. trim., Q > 30	192 M	3-151	45	45.7
Adap. trim., Q > 20, head crop 12 bp	291 M	1-139	44	17.8
Adap. trim., Q > 20, head crop 12 bp, minlen 25 bp	257 M	25-139	44	27.3
Adap. trim., Q > 20, head crop 12 bp, minlen 50 bp	225 M	50-139	44	36.4

De novo transcriptome assembly

When there is no available reference genome for aligning RNA sequencing (RNA-Seq) data, an alternative strategy is to generate a *de novo* transcriptome as a basis for subsequent analysis. The short reads from RNA-Seq represent fragments of longer mRNA sequences. During an assembly, these short reads are assembled *de novo* into contiguous sequences (contigs), which reflect a large portion of the original mRNA transcribed in the organism (Miller et al., 2010). In manuscript II, the goal was to generate a reference transcriptome to which reads from the different experimental conditions could be aligned in order to estimate differential gene expression. Five *de novo* assemblies (Table 11), with different trimming parameters, were generated using Trinity (ver. 2.3.2; Grabherr et al., 2011; Fig. 8)

Table 11. *De novo* transcriptome assemblies from *Acartia tonsa* RNA sequencing data.

A	*de novo* assembly generated from raw sequencing data.
B	*de novo* assembly generated from trimmed data (adapter removal, Q>20, 12 bp head crop) for reads with a minimum length of 25 bp. Trinity were run without the normalization parameter and a contig cutoff of 200 bp.
C	*de novo* assembly generated from trimmed data for reads with a minimum length of 25 bp. Trinity were run with the normalization parameter and a contig cutoff of 200 bp.
D	*de novo* assembly generated from trimmed data for reads with a minimum length of 50 bp. Trinity were run with the normalization parameter and a contig cutoff of 200 bp.
E	*de novo* assembly generated from trimmed data for reads with a minimum length of 50 bp. Trinity were run with the normalization parameter and a contig cutoff of 300 bp.

Trinity, is currently considered to be the most accurate software for *de novo* transcriptome assemblies from NGS data (Haas et al., 2013; Li et al., 2014). Three major modules (Inchworm, Chrysalis, and Butterfly) are the bearing components of Trinity, together with other

dependencies, like Jellyfish and Bowtie (Haas et al., 2013). In short, the assembly pipeline of the Trinity is a build-up of the following modules:

Jellyfish extracts all overlapping kmers from the sequencing data and increases the speed of Trinity (Marçais and Kingsford, 2011).

Inchworm examines each unique kmer in decreasing abundance from Jellyfish and assembles the initial contigs of the transcriptome (Haas et al., 2013). Beginning with the most frequent observed kmer, it examines for kmers shifted one base pair in the 3' direction. For example, if the first kmer is ACCTGT, then it would search for kmers beginning with CCTGT (Haas et al., 2013).

Bowtie is a commonly-used alignment tool that has recently been implemented in the Trinity pipeline for paired-end reads used to "scaffold" contigs together, assuming the paired-reads span across them (Haas et al., 2013; Langmead and Salzberg, 2012).

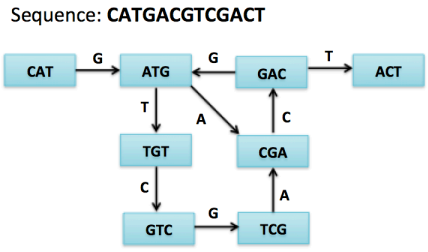
Chrysalis groups related Inchworm contigs and builds a *de Bruijn* graphs (Haas et al., 2013). The *de bruijn* approach creates a directed graph in which nodes represent overlapping kmers (k-1) and edges connecting these overlaps represent the kmers (Compeau et al., 2011). During the assembly, a set of paths covering the graph will be returned, such all the possible assemblies of the data will contain these paths. Each contig represents different paths of the *de bruijn* graph (Compeau et al., 2011). Normalization of kmers (i.e., khmer algorithm) is done during the Trinity assembly (Crusoe et al., 2015; MacManes, 2014; Pell et al., 2012).

Butterfly collapses the *de Bruijn* graphs and aligns the reads to them, distinguishing between splice forms and paralogs (Haas et al., 2013).

The resulting *de novo* transcriptome files (i.e., fasta file) contain a number of assembled contigs representing the mRNA transcripts sequenced from *Acartia tonsa*

De Bruijn Graph

Example of a *de bruijn* graph for a sequence, CATGACGTACT, with kmer = 3. The nodes represents k-1, thus each kmer overlaps by 1 base.



Since smaller contigs tend to show low levels of expression, are of unknown function, or are artefactual (e.g., chimeric transcripts, poor quality, contamination from other species), the contig cutoff was set to 300 bp. It should be noted that not all short contigs are erroneous or useless, but in RNA-Seq pipelines, the focus is on larger patterns of gene expression. Thus, choosing larger contig cutoffs gives higher confidence during annotation and higher statistical power during gene expression analysis.

Validation of de novo transcriptome assembly

There are several approaches to quantitatively and qualitatively comparing transcriptome assemblies.

The N50 statistic is an indicator of assembly quality in terms of contiguity. The N50 value represents the minimum length (in bp) of contigs containing at least 50% of the assembled bases (Martin and Wang, 2011). For example, transcriptome E has a N50 value of 1,874 bp, meaning that at least 50% of the assembled bases are found in contigs that are at least 1,874 bp in length (Table 12). N50 values and minimum average contig lengths of 1,000 bp are recommended as quality parameters (personal communication; Professor Petra Lenz, Pacific Biosciences Research Center, Hawaii, U.S.A.). All five assemblies (Table 12) had N50 values larger than 1,000 bp when the statistics were based on all contigs. If Trinity assemblies have too many contig-isoforms, the N50 values can be overestimated and the N50 value should be based on the single longest isoform per "gene". It should be noted that Trinity "genes" refer to clusters of similar contigs and not actual genes. For the N50 statistics, based on the longest isoform per "gene", all assemblies had N50 values above 1,000 bp, except transcriptome A (i.e., raw reads assembly) (Table 12). N50 statistics can be biased either by being skewed towards higher N50 values, if Trinity generates many different long isoforms for a gene, or skewed towards lower N50 values if Trinity reconstructs contigs for transcripts with low expression, which tend to be short. Thus, N50 statistics cannot be used as stand-alone for validating assembly quality.

Table 12: Validation of following 5 *de novo* transcriptome assemblies generated with Trinity (ver. 2.3.2; Grabherr et al., 2011): **A)** *de novo* assembly generated from raw sequencing data. **B)** *de novo* assembly generated from trimmed data (adapter removal, Q>20, 12 bp head crop) for reads with a minimum length of 25 bp. Trinity was run without the normalization parameter and a contig cutoff of 200 bp. **C)** Normalized Trinity assembly from trimmed reads (as in B), contig cutoff = 200. **D)** Normalized Trinity assembly from trimmed reads, as in B), but with a minimum read length of 50 bp, contig cutoff = 200. **E)** Normalized Trinity assembly from trimmed reads (as in C), contig cutoff = 300. "Trinity" is the summary statistics, with the number of Trinity components or "genes" (#genes), number of transcripts (#transcripts) and the GC content (%GC). The N50 statistics were done based on all contigs (Contigs) and longest isoform per "gene" (Isoform) giving the N50 value, average contig length and the number of assembled bases (# assembled bases) (Trinity, ver. 2.3.2; Grabherr et al., 2011). E90N50 is when at least 90% of the assembled bases are in contigs of at least the E90N50 value length given in bp (Trinity, ver. 2.3.2; Grabherr et al., 2011). A total of 303 BUSCOs (Benchmarking Universal Single-Copy Orthologs, ver. 2; Simão et al., 2015), were searched to examine completeness the assemblies based on % complete BUSCOs (C), % complete and single-copy BUSCOs (S), % complete and duplicated BUSCOs (D), % fragmented BUSCOs (F), and missing BUSCOs (M). Bowtie2 (ver. 2.2.6; Langmead and Salzberg, 2012) was used to realign input sequencing reads to the assemblies to examine contig contiguity in terms of read representation (% alignment). The DETONATE score (DE novo TranscriptOme rNa-seq Assembly with or without the Truth Evaluation, ver. 1.11; Li et al., 2014) are used to compare assemblies. The highest DETONATE score gives an indication on the best assembly. The Transrate score (ver. 1.0.3; Smith-Unna et al., 2016) were also, like DETONATE, used to compare and evaluate transcriptome assemblies. The score ranged from 0 to 1, where the assemblies closest to 1 are considered the best. Transrate scores are, however, biased towards large numbers of low-expressed transcripts.

Parameter		**A**	**B**	**C**	**D**	**E**
Trinity	*# "genes"*	109,352	48,501	27,102	48,744	27,171
	# transcripts	153,899	86,347	61,068	86,426	60,688
	% GC	36.38	38.13	38.50	38.17	38.49
Contigs	*N50 (bp)*	1,027	1,736	1,876	1,733	1,874
	Average contig length (bp)	627.79	928.07	1,230.89	926.03	1,222.45
	# assembled bases	96,615,572	80,135,880	75,168,266	80,032,649	74,188,026
Isoform	*N50 (bp)*	707	1,510	1,871	1,478	1,861
	Average contig length (bp)	527.95	714.41	1,089.67	708.07	1080.47
	# assembled bases	57,732,838	34,649,358	29,532,360	34,514,076	29,357,348
E90N50 (bp)		2,921	2,737	2,752	2,707	2,731
ExN50 (bp)		E65N50: 3,187	E68N50: 3,100	E71N50: 3,144	N68E50: 3,026	E69N50: 3,075
BUSCO		C:98.6% S:63.0% D:35.6% F:0.7% M:0.7%	C:98.6% S:46.5% D:52.1% F:0.7% M:0.7%	C:98.3% S:44.2% D:54.1% F:1.0% M:0.7	C:99.0% S:46.2% D:52.8% F:0.7% M:0.3%	C:99.0% S:44.9% D:54.1% F:0.7% M:0.3%
Bowtie2		91.17%	90.92%	90.35%	91.10%	90.35%
DETONATE		-222347343 38	-219197137 50	-218977105 02	-218770722 56	-218345240 62
Transrate		0.01475	0.01296	0.01115	0.01293	0.01191
SortMeRNA		2.86%	1.40%	1.73%	1.29%	1.61%

The N50 statistic gives information about contiguity, but the additional ExN90 statistic is more appropriate, since it represents the most frequent contigs (90%) and also provides additional information about whether the sequencing depth was sufficient (Fig. 9). ExN90 indicates that at least 90% of the assembled bases are in contigs of at least the given ExN90 length (in bp). For transcriptome E (Fig. 9, Table 12), 90% of the assembled bases are in contigs that are at least 2,731 bp in length. When plotting the ExN90 (bp) versus E (%) values, it is preferable to observe a peak in ExN90 at an E-value of 90 suggesting the optimal sequencing depth. At insufficient sequencing depths, the graphed line will peak and flatten at lower E values. As read depth increases, the ExN50 peak will shift towards ~90%. The five assemblies (Fig. 9, Table 12) all peak around 70%. Deeper sequencing could bring the peak closer to 90% and provide higher quality assemblies, but the necessary balance between yield and cost may prevent deeper sequencing, but the peak around 70% is considered acceptable.

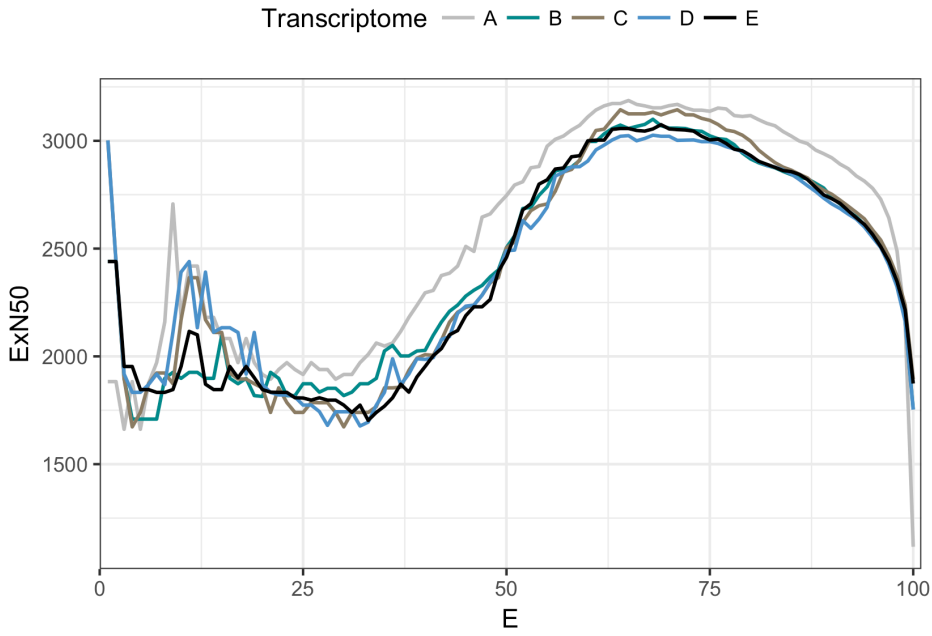


Figure 9. ExN50 profile for following 5 *de novo* transcriptome assemblies generated with Trinity: **A)** *de novo* assembly generated from raw sequencing data. **B)** *de novo* assembly generated from trimmed data (adapter removal, Q>20, 12 bp head crop) for reads with a minimum length of 25 bp. Trinity was run without the normalization parameter and a contig cutoff of 200 bp. **C)** Normalized Trinity assembly from trimmed reads (as in B), contig cutoff = 200. **D)** Normalized Trinity assembly from trimmed reads, as in B), but with a minimum read length of 50 bp, contig cutoff = 200. **E)** Normalized Trinity assembly from trimmed reads (as in C), contig cutoff = 300. E represents the percentage of contigs that are at least of the ExN50 value length (in bp).

The completeness of the five assemblies was examined using the Benchmarking Universal Single-Copy Orthologs (BUSCO, ver. 2; Simão et al. 2015), which defines a set of eukaryotic core genes (N=303) used to test the proportion and completeness of these genes in the corresponding transcriptome assembly. The two most complete assemblies were transcriptomes D and E, with 99.0% complete BUSCOs (Table 12). Among a total of 3030 BUSCO groups searched, transcriptome D had 46.2% complete and single-copy BUSCOs (S), 52.8% complete and duplicated BUSCOs (D), 0.7% Fragmented BUSCOs (F), and 0.3% Missing BUSCOs (M). Transcriptome E had same percentage of (F) and (M), but differed with 44.9% (S) and 54.1% (D) (Table 12). The BUSCO scores indicated high completeness in terms of the searched orthologs for all five assemblies.

Paired-end reads are not always properly represented in the assembled transcriptome, because they might be fragmented or short or because only one of a pair of reads aligns. The quality and contig contiguity in terms of read representation were evaluated by realigning the input sequencing reads to each of the five assemblies using Bowtie2 (ver. 2.2.6; Langmead and Salzberg, 2012). Ideally, at least 70% of the sequencing reads should be represented in the transcriptome (Conesa et al., 2016). Low alignment is likely to result from transcripts with low expression that have insufficient coverage or low-quality reads. A total of 90.35% to 91.17% of the sequencing reads was represented when realigned to the assemblies (Table 12).

The transcriptomes were also evaluated using two additional software packages that calculate scores for each assembly based on overall quality.

First, the DETONATE (DE novo TranscriptOme rNa-seq Assembly with or without the Truth Evaluation) package RSEM-EVAL (ver. 1.11, Li et al., 2014) was used to evaluate *de novo* transcriptome assemblies with a probabilistic model using the sequencing reads and the assembly itself. In contrast to N50 and ExN50 statistics, RSEM-EVAL combines multiple factors (e.g., compactness of assembly, support of the assembly from the sequencing reads) into a single negative evaluation score (Li et al., 2014). The less negative DETONATE score, the better is the quality of the assembly (Li et al., 2014). The DETONATE score for transcriptome E is the least negative one, thus suggested as the best assembly among the five (Table 12).

Second, Transrate was used to compute evaluation scores (between 0 and 1) based on assembly errors (e.g., chimeras, structural errors, incomplete assembly, base errors; Smith-Unna et al., 2016). The assembly with the highest Transrate score is considered the best transcriptome (Smith-Unna et al., 2016). Transrate is, however, biased towards transcripts with low expression. The assemblies all exhibited Transrate scores below 0.02 (Table 12), with transcriptome A achieving the highest value. This can be explained partly by the high sequencing depth of ~350 M reads, which will include transcripts with low expression and short contigs. DETONATE is, thus, a better approach for evaluating the overall assembly quality.

Since rRNA can comprise most of the total RNA, various pre-sequencing procedures are used to either deplete rRNA or enrich mRNA. Even though these procedures are effective, they do not always eliminate the rRNA. To avoid down-stream issues with carryover rRNA, SortMeRNA (ver. 2.1, Kopylova et al., 2012) was used to identify and remove rRNA sequences. All assemblies contained low amounts of rRNA (< 3%) suggesting that the library prep was successful in removing most of the rRNA.

When validating assemblies, it is desirable to have high N50 values and average contig lengths, preferably above 1,000 bp. Transcriptome A, which was constructed from raw sequencing reads, was expected to be of lowest quality, due to lack of quality trimming and inclusion of short reads in the assembly. The average contig length of transcriptome A did not exceed the recommended threshold of 1,000 bp when based on all contigs or on the longest isoform per "gene" (Table 12). The same applied to transcriptome B, which was constructed from trimmed sequencing data (adapter removal, Q > 20, 12 bp head crop) with the minimum read-length of 25 bp. The transcriptome B assembly was performed without kmer normalization, and had a contig cutoff of 200 bp (Table 12). Transcriptome C was constructed in the same way as Transcriptome B, with the exception of kmer normalization during the *de novo* assembly. The additional normalization resulted in an average contig length above 1,000 bp for transcriptome C, which had the best N50 statistics in terms of N50 values (in comparison to the four other assemblies) and average contig lengths (Table 12). The N50 statistics of transcriptome C were, however, very similar to transcriptome E (Table 12). Transcriptome D was constructed in the same way as transcriptome C, except that reads with a minimum length of 50 bp, instead of 25 bp, were used for the assembly. The average contig length for transcriptome D (based on all contigs and longest isoform per "gene") was below the 1,000 bp threshold, caused by a high number of shorter contigs (e.g., 200-300 bp; Table 12). The contig cutoff of 300 bp for transcriptome E removed the high number of shorter contigs observed in transcriptome D, resulting in an average contig length that exceeded the threshold of 1,000 bp, as well as higher N50 values (Table 12). All five assemblies (i.e., A, B, C, D, and E) had N50 values above the recommended 1,000 bp threshold (Table 12).

Based on BUSCO analysis, transcriptomes D and E were the most complete among the assemblies, with a total of 99.0% complete BUSCOs (Table 12). Transcriptome C exhibited the lowest degree of completeness, with a total of 98.3% BUSCOs (Table 12). All assemblies did, however, exhibit a very high degree of completeness in terms of the BUSCO analysis.

Realignment of input sequencing reads to the *de novo* transcriptome assemblies resulted in ~90% alignment (Table 12). This indicates that the reads were well-represented in all of the assemblies, with alignments above the recommended threshold of 80%.

Transcriptome A had the highest Transrate score, followed by transcriptome B (Table 12). This suggests that the analysis was biased against a large number of reads with low expres-

sion and short contigs. Transcriptomes C and E had the lowest Transrate scores, probably due to fewer shorter contigs, which tend to exhibit low expression levels.

Based on the overall picture given by the validation approaches used, transcriptome E was selected as the reference transcriptome for manuscript II. Transcriptome E had acceptable N50 and ExN50 values, as well as the best DETONATE score. Some of the quality parameters were, however, very similar to transcriptome C. The shorter contigs of transcriptome C would "contaminate" the assembly and affect subsequent differential gene expression analysis by lowering the statistical power. Transcriptome E was therefore considered a better choice as the reference assembly.

Differential gene expression

Traditional methods (e.g., the Tuxedo pipeline) for estimating differential gene expression involve the alignment of experimental sequencing reads to a reference genome or transcriptome, followed by estimation of transcript abundance (Conesa et al., 2016; Oshlack et al., 2010). In addition to being very time-consuming and requiring high computational capacity, non-model organisms often lack reference genomes (Bray et al., 2016; Pimentel et al., 2017). Recent advances in bioinformatics have resulted in "reference-free" methods (e.g., Sailfish, Kallisto, Salmon), in which the transcriptomes are shredded into kmers. Experimental sequencing of kmers can then be matched to the kmers from the transcriptome, resulting in fast and accurate estimations of abundance (Conesa et al., 2016; Soneson et al., 2016). Kallisto (ver. 0.43.0, Bray et al., 2016), which was used for estimating transcript abundance in manuscript II (Fig. 8), deviates slightly from the other kmer-shredding approaches (e.g., Sailfish, Salmon etc.) by using pseudo-alignment. Kallisto constructs a *de Bruijn* graph from transcriptome kmers (i.e., t-DBG; Bray et al., 2016). During pseudo-alignment, the kmers from experimental reads are evaluated for compatibility with the t-DBG (Bray et al., 2016). By skipping kmers for whichcompatibility does not change with the t-DBG, the process is accelerated. Kallisto only accounts for exact kmer matches, and therefore discards sequencing errors (i.e., kmers not compatible with t-DBG) (Bray et al., 2016). The additional bootstrapping is another strength of Kallisto, since the focus is then solely on the biological, rather than the technical, variance of the experimental samples (Bray et al., 2016).

Abundances estimated using Kallisto are reported in Transcripts Per Million (TPM) rather than RPKM (Reads Per Kilobase Million) or FPKM (Fragments Per Kilobase Million) (Bray et al., 2016). TPM is normalized for mean expressed transcript length, so the sum of TPMs will be the same for each sample, making it easier to compare the proportion of reads mapped to a gene (Conesa et al., 2016; Das et al., 2016). The sum of normalized reads for RPKM and FPKM may differ between samples, making it more difficult to compare samples (Conesa et al., 2016; Das et al., 2016).

The R package, Sleuth, is designed for processing and analyzing output abundance data from Kallisto (Pimentel et al., 2016). Sleuth is able to perform expression analysis at both the transcript and gene level (Pimentel et al., 2016).

In manuscript II, Sleuth was used to perform expression analysis for both transcripts and genes. Differentially expressed transcripts that were not annotated by the Trinotate pipeline (Fig. 8) were isolated and annotation ws attempted using Blast2Go. The additional annotations from Blast2Go were included in a final expression analysis at the gene level (Fig. 8).
Sleuth uses flexible-response error models (e.g., Wald and LRT) that allowdifferent experimental designs, and also remove technical variance in the samples based on Kallisto bootstrapping (Pimentel et al., 2016).
Instead of a log fold-change, used in other approaches (e.g., DeSeq2, EdgeR), Sleuth computes a beta-value (i.e., b-value) related to the Wald test. The b-value can be defined as a bias estimator of the fold-change, that is the extent to which the transcript count is affected by the experimental treatment rather than technical variability (Pimentel et al., 2016). The less noise and variability observed for a transcript, the closer it will be to its actual fold-change. If the variability of the transcript is high, the b-value will be more conservative and account for less of the overall estimated count, even though the fold-change may be higher (Pimentel et al., 2016). Where other approaches (e.g. DeSeq2) tend to underestimate the false discovery rate (FDR), Sleuth is more conservative and tends to overestimate the FDR, reporting fewer genes that are highly enriched by being differentially expressed (Pimentel et al., 2016). The approach is more conservative because it uses q-values, rather than p-values, which include an adjusted p-value accounting FDR of 0.01 (Pimentel et al., 2016).
The Kallisto-Sleuth pipeline is thus considered one of the best for estimating differential expression because the pseudo-alignment procedure is robust in response to erroneous sequencing reads and the statistical approach (i.e., Sleuth) has a conservative nature (Bray et al., 2016; Sahraeian et al., 2017).
Gene Ontology (GO) provides information about gene function (ontologies) for three categories of gene products: biological function, cellular compartment and molecular function (Ashburner et al., 2000). In manuscript II, gene enrichment analyses were performed in order to discover common characteristics of differentially expressed genes in response to handling stress and salinity shock. GO enrichment analysis was conducted using the R package, ClusterProfiler (ver. 3.6.0, Yu et al., 2012).

Main findings and discussion

The research presented here considered numerous aspects of stress responses at the transcriptional level in the copepod, *Acartia tonsa*. The main findings of the research are discussed in the following:

Copepods do not experience stress during short-term elevated densities up to 10,000 ind. L^{-1}.

It has been hypothesized that *A. tonsa* is difficult to rear under high-density conditions, suggesting that stress might have an influence (e.g., Franco et al., 2017). The copepods didnot, however, demonstrate any stress response to short-term (i.e., 12 h) high-density (up to 10,000 ind. L^{-1}) conditions, in terms of swimming behavior, respiration rate or gene expression levels of *ferritin* or *heat-shock protein 70 or 90 kDa* (*HSP70*, *HSP90*). Other genes could potentially respond at the transcriptional level. But since no significant changes were observed in any of the other end-points (i.e., swimming behavior and respiration rate), we conclude that *A. tonsa* does not experiencing stress under these conditions.

The concerns about rearing *A. tonsa* at high-densities may be due either to the copepods experiencing stress over longer time-periods (i.e., chronic rather than acute stress) or to food shortage. The question about what is the real "high-density carrying capacity" remains and needs further studies. It could for instance be interesting examining the transcriptional response (targeted – or transcriptome-wide) over the course of one – or even multiple generations.

The *A. tonsa* transcriptome gives the possibility of new biomarkers and reference genes.

Before producing the *de novo* transcriptome in manuscript II, it was challenging to examine stress responses at the transcriptional level in *A. tonsa*. The number of potential reference and biomarker gene sequences was very limited. The process of producing new biomarkers for stress-related studies was challenging, since the production of degenerate primer sequences was very time-consuming and not always successful. The *A. tonsa de novo* transcriptome opened up the possibility of mining for new biomarkers and reference genes for future studies, as demonstrated in manuscript III.

Validation of old and new reference genes.

Using previously-known reference genes for *A. tonsa*, as well as mining for new genes in the *de novo* transcriptome, seven potential reference genes (i.e., *ACT*, *EFA*, *ATPS*, *S20*, *HIST*, *18S* and *UBI*) were examined for samples from experimental culture conditions, as described in the three manuscripts. Based on the initial findings for the geNorm analysis (Perkins et al., 2012; Vandesompele et al., 2002), *HIST* and *ATPs* were selected for normalization of gene expression in manuscript III. The Ct values of *HIST* and *ATPs* across all

the experimental samples in manuscript III were stable, thus normalization used these two. The additional analysis with BestKeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004), as well as the pairwise variation analysis with geNorm (Perkins et al., 2012; Vandesompele et al., 2002), suggests that four reference genes are optimal for normalization of expression levels in *A. tonsa* (i.e. *HIST*, *UBI*, *S20*, and *ATPS;* top-ranked candidate genes across all three analyses.) across very different life-stages and varied conditions. Since manuscript III only concerned embryos, and the Ct-values of *ATPS* and *HIST* were stable across conditions, normalization using these two were sufficient. If normalization against the two selected reference genes weren't sufficient, it would be reflected in a larger standard deviation of the data represented in Fig. 3, manuscript III. But since these variations are within an acceptable range, normalization using only *ATPS* and *HIST* was applicable.
In addition, *18S* should not be used as reference gene, due to its very high expression level compared to the other reference genes, which has also been observed for the copepod, *Calanus sinicus* (Lauritano et al., 2015). This suggests that *18S* is inappropriate as a copepod reference gene.

Handling affects transcriptome-wide gene expression, so the choice of biomarkers and timing of preservation after exposure to stress are important.
Gene expression of selected biomarkers is commonly used to examine how stressors affect copepods at the transcriptional level (e.g., Lauritano et al., 2015). Experimental handling is also potential an experimental condition in animal studies and can potentially affect the expression of biomarkers, leading to false interpretation of the results. Using RNA sequencing (RNA-Seq), the transcriptome-wide response to handling stress and salinity shock was examined in *A. tonsa* at both 15 min and 24 h after exposure. Very few differentially expressed genes were observed 15 min after exposure to either handling stress or salinity shock. In contrast, hundreds of differentially expressed genes were observed for both treatments 24 h after exposure. These results indicate that copepods, and potentially other zooplankton species, should be preserved quickly, preferably within 15 min, to avoid the transcriptional effects of handling when performing gene expression studies.
It is important to be aware of handling when examining stress at the transcriptional level, regardless of the animal species in question, since gene expression potentially can be affected by handling. This should also be considered when analysis uses selected biomarkers and targeted-gene approaches, which may also be sensitive to handling stress.

Genes related to the unfolding protein response (i.e., ER-homeostasis related genes, *ORNT*, *CUBN*), chaperone-mediated protein folding (e.g., heat shock proteins, *TCPG*, *FKBP4*, *FKBP*), detoxification (e.g., *GST1, QCR2, CP2J2, CP18A, ALDH*), and apoptosis (i.e., ubiquitin and related genes; *UHRF1*, *UBE2C*, *NDUV2*, *UBR4*, *UBP25, FANCL*) were found among the differential expressed genes in exposure to handling and salinity shock (manuscript II, appendix I).
It is problematic since some of these genes, that are differential expressed in relation to handling, also are being used as transcriptional biomarkers in copepod studies (Table 1). The choice of suitable transcriptional biomarkers is, thus, a complicated task. The genes

need to be sensitive, in terms of response, towards the stressor in question. But even if the stressor is the same in multiple transcriptional studies, variation will occur depending upon the test species employed, how the species are acclimatized to different environments, and the life-stages and general fitness of the individuals used in the experimental manipulations. In addition to differences caused by the stressor and the organism targeted, comparisons among studies are further complicated by inconsistencies in the experimental designs. This includes different handling methods and sampling times, which may generate different transcriptional response patterns. We are fortunate that the *A. tonsa* strain used in this study has been cultured intensively under the same conditions for years, which allows stronger comparisons between studies using this particular strain.

In targeted transcriptional approaches, the choice of biomarkers should be well considered, and suitable controls on experimental handling should be included, to ensure that the observed expression of the chosen biomarker genes is not affected by handling stress. Because of differences of test organisms, stressors and experimental procedures, transcriptional patterns between studies can be difficult to compare directly.

The first approach to understanding transcriptome-wide effects of handling.
For many genes, it is unknown whether and when a response can be detected at the transcriptional level. When an organism is exposed to and needs protection from a certain stressor, some genes will be up-regulated quickly after exposure to prevent damage. These include elements of various signaling pathways for initiating stress responses (de Nadal et al., 2011). Other genes will be up-regulated later for helping "clean up the mess" of damaged proteins and for restoration of homeostasis (Goldstein and Kopin, 2007). For instance, *HSP70* peaks a few hours after stress-exposure in *A. tonsa* (Aruda et al., 2011; Petkeviciute et al., 2015; Rahlff et al., 2017). In manuscript II, the focus was on analysis of copepods at 15 min and 24 h after exposure, in order to answer the question: when is a response detectable? The results indicated that a potential peak of *HSP70* was not detected for either handling stress or salinity shock treatments. Furthermore, transcriptional patterns largely depend on the species in question. Even for same species, the transcriptional patterns can vary because of acclimation to different environments and "lifestyle" (e.g., Aguilera et al., 2016). The 10 min of exposure to handling and salinity stress may be extreme in relation to some laboratory experiments. But since this is the first approach to understanding how handling stress affects gene expression, it was important to ensure that the response was detectable at the transcriptomic level. Hereafter, studies will be able to fine-tune the approach and select biomarkers of interest for analysis of responses to less extreme handling conditions and salinity shock.
When the AMPHICOP effort of producing an *A. tonsa* genome is finalized, it is possible to re-analyze the data by mapping them to the genome. This may give new information in addition to what is presented in manuscript II. Furthermore, it may also improve the functional annotation and enrichment analysis, since the annotations in manuscript II were based on model organisms like human, mouse, and fruit fly.

Inconsistency between transcriptional studies makes them difficult to compare directly.

Studying stress responses at the transcriptional level is complicated by a number of factors, including the target species, life stages, acclimatization/acclimation, and many others. Even individuals of then same species can have different transcriptional patterns, depending on their lifestyle (e.g., Aguilera et al., 2016). In addition, the time needed to detect a transcriptional stress response may vary among genes. For instance, gene expression of *HSP70* peaked within a few hours after stress exposure. This was not detected in manuscript II, because the measurements were performed 15 min and 24 h after exposure. Thus, differences in experimental procedures, incubation periods, methods of handling the organisms, and handling duration are all factors with the potential of affecting gene expression patterns, which makes it difficult to directly compare studies (Havird and Santos, 2016; Mykles et al., 2016). It is, however, beneficial that the strain of *A. tonsa* used for this analysis is well-studied, with the same stable conditions used across numerous studies (e.g., Nilsson et al., 2013; Franco et al., 2017). This makes comparison far easier than comparing two different crustacean species, which may be acclimatized to different environments, have different lifestyles, and thus different fitness in relation to the stressor being tested. Establishing a set of best practices for experimental design, analysis of gene expression, and bioinformatics can minimize the effects of inconsistency among studies (Havird and Santos, 2016; Mykles et al., 2016).

The first detailed description of subitaneous development and quiescence in *A. tonsa* eggs.

Stressful conditions are able to induce the dormant state, quiescence, during *A. tonsa* embryonic development (e.g., Drillet et al., 2011; Nilsson et al., 2013; Hansen et al., 2016). In manuscript II, the first detailed description of subitaneous development and quiescence in *A. tonsa* eggs are given. Eggs that had not developed further than gastrulation during subitaneous development would stay in gastrulation during quiescence. How far the eggs were in the subitaneous development before inducing quiescence had an impact on later hatching success.

Furthermore, analysis of expression of genes involved in the ecdysone-signaling pathway suggested that embryonic molting during subitaneous development is regulated by ecdysteroids, as found for other arthropods (e.g., Warren et al., 1986; Chávez et al., 2000; Kozlova and Thummel, 2003).

Conclusions and perspectives

The following conclusions and perspectives can be drawn from the studies described in this dissertation:

- *Acartia tonsa* do not experience stress in response to short-term elevated densities, in terms of swimming behavior, respiration rates, and gene expression analysis of *ferritin*, *heat-shock protein 70* – and *90 kDa*. Transcriptome-wide approaches (e.g., RNA sequencing) and other biomarkers may give more information about why *A. tonsa* are so difficult to cultivate in high densities.
- Four reference genes are suggested for normalization of targeted gene expression levels in *A. tonsa*. *HIST*, *ATPS*, *S20* and *UBI* have been suggested, due to their stable expression across the different treatments described in the three studies of this dissertation. As a reference gene, *18S* should be avoided because of its high expression in relation to the other tested reference genes.
- Handling stress affects gene expression of *A. tonsa*. Biomarkers should be carefully selected for studies environmental stress. Handling should in general be considered as a factor that is able to influence gene expression, regardless of the animal species in question.
- Timing is essential when it comes to handling. Copepods, and possibly other zooplankton species, should preferably be preserved within 15 min after collection to avoid influences on gene expression from experimental handling.
- Responses to handling stress and salinity shock, in terms of gene expression (e.g., *HSP70*), peak between 15 min and 24 h after exposure. Changing the durations of the 10 min exposure, as well as considering the post-exposure period, could target areas for future studies, in order to achieve a better understanding of stress responses in copepods.
- The reference transcriptome described in manuscript II opens possibilities for identification of new biomarkers and reference genes for *A. tonsa* in future studies.
- Manuscript III provides the first detailed description of subitaneous development and quiescence in *A. tonsa* eggs. The timing of induction of dormancy affects hatching success.
- Understanding of the epigenetic mechanisms underlying embryonic dormancy in copepods (i.e., quiescence, diapause and delayed hatching) may be of interest for future studies.
- The transcriptomic approaches of the studies described in this dissertation, as well as additional work carried out during AMPHICOP to generate a reference genome for *A. tonsa* bring it closer to functioning as a model species.

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Appendix I

Overlapping differential expressed genes, 24 h post exposure to handling and salinity shock. b: beta-values; SE: standard errors of beta-values; q-values: false discovery rate adjusted p-values

Symbol	Gene	Handling 24 h post-exposure			Salinity shock 24 h post-exposure		
		b	SE	q-value	b	SE	q-value
ZN865	Zinc finger protein 865	4.12	1.10	0.0068451	4.35	1.10	0.0027984
N4BP1	NEDD4-binding 1 isoform X1	3.85	1.13	0.0163692	3.22	1.13	0.0476691
LOC105360031	LOC105360031	3.56	0.48	0.0000000	4.08	0.48	0.0000000
LOC108673941	LOC108673941	3.49	0.69	0.0000581	3.95	0.69	0.0000018
LOC105689904	LOC105689904	3.29	0.91	0.0090886	3.74	0.91	0.0016064
TCPG	T-complex protein 1 subunit gamma	3.16	0.59	0.0000143	3.39	0.59	0.0000015
YFKF	uncharacterized MFS-type transporter -like	2.91	0.86	0.0177548	2.91	0.86	0.0133761
S19A3	Thiamine transporter 2	2.53	0.34	0.0000000	2.76	0.34	0.0000000
TMM53	Transmembrane protein 53	2.47	0.65	0.0062645	2.55	0.65	0.0032228
NCED1	9-cis-epoxycarotenoid dioxy-genase 1, chloroplastic	2.40	0.49	0.0001317	3.19	0.49	0.0000000
MOB2	MOB kinase activator-like 2	2.38	0.58	0.0024779	2.54	0.58	0.0006851
PPP1R11	phosphatase 1 regulatory subunit 11	2.35	0.78	0.0417863	2.68	0.78	0.0114646
CDPF1	Cysteine-rich DPF motif do-main-containing protein 1	2.27	0.44	0.0000405	2.42	0.44	0.0000063
DEN1A	DENN domain-containing protein 1A	2.09	0.34	0.0000005	2.27	0.34	0.0000000
ZHX2	Zinc fingers and homeoboxes protein 2	2.01	0.56	0.0095338	2.13	0.56	0.0040962
KNRL	Knirps-related protein	1.97	0.35	0.0000052	2.09	0.35	0.0000005
FABD	Malonyl-CoA-acyl carrier pro-tein transacylase, mitochon-drial	1.93	0.40	0.0001796	2.25	0.40	0.0000033
UBP25	Ubiquitin carboxyl-terminal hydrolase 25	1.90	0.50	0.0058374	2.42	0.50	0.0001023
OPN5	Opsin-5	1.88	0.43	0.0008590	2.24	0.43	0.0000170
ESCO2	N-acetyltransferase ESCO2	1.88	0.43	0.0008590	2.22	0.43	0.0000211
SPRY3	SPRY domain-containing pro-tein 3	1.74	0.32	0.0000117	2.38	0.32	0.0000000
SAP	Prosaposin	1.74	0.48	0.0091534	1.81	0.48	0.0046941
CPTP	Ceramide-1-phosphate trans-fer protein	1.47	0.34	0.0008590	1.71	0.34	0.0000279
Y2049	Uncharacterized oxidoreduc-tase SERP2049	1.38	0.35	0.0038386	1.75	0.35	0.0000469
QCR2	Cytochrome b-c1 complex subunit 2. mitochondrial	1.37	0.19	0.0000000	1.80	0.19	0.0000000
PCGF3	Polycomb group RING finger protein 3	1.30	0.39	0.0185863	1.31	0.39	0.0129691
DISP1	Protein dispatched homolog 1	1.30	0.28	0.0002381	1.10	0.28	0.0024788

FKBP4	Peptidyl-prolyl cis-trans isomerase FKBP4	1.29	0.26	0.0000799	1.36	0.26	0.0000174
ORNT1	Mitochondrial ornithine transporter 1	1.19	0.25	0.0002381	1.81	0.25	0.0000000
HDAC1	Histone deacetylase 1	1.19	0.33	0.0083885	1.10	0.33	0.0136984
SNIP1	Smad nuclear-interacting protein 1	1.18	0.18	0.0000000	1.15	0.18	0.0000001
CPR1	Putative cysteine proteinase CG12163	1.18	0.38	0.0363557	1.43	0.38	0.0052884
FKBP5	Peptidyl-prolyl cis-trans isomerase FKBP5	1.07	0.22	0.0001773	1.05	0.22	0.0001679
LOC108772247	LOC108772247	1.07	0.26	0.0025215	0.85	0.26	0.0170218
BUB3	Mitotic checkpoint protein BUB3	0.97	0.32	0.0387830	0.98	0.32	0.0267725
LAML3	Lamin-L(III)	0.94	0.19	0.0000541	0.99	0.19	0.0000111
SMG5	Protein SMG5	0.91	0.29	0.0329189	1.23	0.29	0.0010012
POLY	Retrovirus-related Pol polyprotein from transposon gypsy	0.90	0.28	0.0276536	1.35	0.28	0.0001078
Hel25E	ATP-dependent RNA helicase WM6	0.89	0.24	0.0062973	0.72	0.24	0.0284388
CHM1B	Charged multivesicular body protein 1b	0.80	0.26	0.0398120	0.86	0.26	0.0158790
CHIT2	Probable chitinase 2	0.74	0.24	0.0314687	1.01	0.24	0.0009299
TEN3	Teneurin-3	0.72	0.22	0.0183985	1.05	0.22	0.0000863
LOC103313083	LOC103313083	0.71	0.18	0.0030982	0.70	0.18	0.0026768
NU155	Nuclear pore complex protein Nup155	0.70	0.15	0.0002753	0.48	0.15	0.0205105
ISY1	Pre-mRNA-splicing factor ISY1 homolog	0.69	0.20	0.0156390	0.64	0.20	0.0219141
DAPK	Death-associated protein kinase dapk-1	0.69	0.16	0.0008590	0.95	0.16	0.0000005
MYLK	Myosin light chain kinase. smooth muscle	0.69	0.16	0.0019619	0.56	0.16	0.0126655
ZG20	Gastrula zinc finger protein xFG20-1	0.67	0.20	0.0167678	0.72	0.20	0.0065837
PP4R2	Serine/threonine-protein phosphatase 4 regulatory subunit 2	0.66	0.21	0.0363557	0.93	0.21	0.0007121
NUP62	Nuclear pore glycoprotein p62	0.65	0.17	0.0064012	0.50	0.17	0.0469893
PP1B	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	0.62	0.09	0.0000000	0.59	0.09	0.0000000
SCNA	Sodium channel protein para	0.62	0.11	0.0000047	0.65	0.11	0.0000006
BAT1	b(0.+)-type amino acid transporter 1	0.61	0.09	0.0000000	0.60	0.09	0.0000000
DOM	Helicase domino	0.60	0.19	0.0369272	0.79	0.19	0.0018587
ACSF3	Acyl-CoA synthetase family member 3. mitochondrial	0.60	0.12	0.0000380	0.40	0.12	0.0104109
MBD3	Methyl-CpG-binding domain protein 3	0.59	0.11	0.0000249	0.58	0.11	0.0000211
PIGC	Phosphatidylinositol N-acetylglucosaminyltransferase subunit C	0.59	0.10	0.0000014	0.53	0.10	0.0000120
SPRY7	SPRY domain-containing protein 7	0.59	0.19	0.0395465	0.84	0.19	0.0007121

JMJD8	JmjC domain-containing protein 8	0.56	0.16	0.0137592	0.60	0.16	0.0053300
EGF	Pro-epidermal growth factor	0.55	0.15	0.0076313	0.49	0.15	0.0147791
INT7	Integrator complex subunit 7	0.54	0.17	0.0345485	0.70	0.17	0.0023731
MTMRD	Myotubularin-related protein 13	0.54	0.09	0.0000008	0.52	0.09	0.0000011
SH2B1	SH2B adapter protein 1	0.53	0.10	0.0000450	0.43	0.10	0.0015848
BN3D2	Pre-miRNA 5'-monophosphate methyltransferase	0.53	0.10	0.0000117	0.41	0.10	0.0010879
FANCL	E3 ubiquitin-protein ligase FANCL	0.53	0.12	0.0004185	0.58	0.12	0.0000417
CB042	Uncharacterized protein C2orf42 homolog	0.53	0.14	0.0082654	0.75	0.14	0.0000183
SETD2	Histone-lysine N-methyltransferase SETD2	0.51	0.16	0.0329113	0.65	0.16	0.0026453
TAMO	Protein tamozhennic	0.51	0.11	0.0002208	0.61	0.11	0.0000024
TM205	Transmembrane protein 205	0.50	0.12	0.0012722	0.51	0.12	0.0006783
MAON	NAD-dependent malic enzyme 59 kDa isoform. mitochondrial	0.49	0.10	0.0002208	0.85	0.10	0.0000000
P27K	27 kDa glycoprotein	0.49	0.09	0.0000080	0.56	0.09	0.0000001
TGFA1	Transforming growth factor-beta receptor-associated protein 1	0.48	0.09	0.0000277	0.39	0.09	0.0010405
CUED1	CUE domain-containing protein 1	0.48	0.11	0.0009924	0.42	0.11	0.0045352
YIPF1	Protein YIPF1	0.47	0.16	0.0393979	0.47	0.16	0.0319679
TTN	titin homolog isoform X1	0.47	0.11	0.0022624	0.36	0.11	0.0219661
HPCD	3-hydroxypropionyl-coenzyme A dehydratase {ECO:0000303|PubMed:19429610}	0.47	0.13	0.0064403	0.38	0.13	0.0313504
LOLA3	Longitudinals lacking protein. isoforms A/B/D/L	0.47	0.09	0.0000293	0.41	0.09	0.0002931
CP2J2	Cytochrome P450 2J2	0.46	0.09	0.0001101	0.49	0.09	0.0000164
GGT1	Gamma-glutamyltranspeptidase 1	0.46	0.09	0.0000293	0.32	0.09	0.0076508
BD1L1	Biorientation of chromosomes in cell division protein 1-like 1 {ECO:0000305}	0.45	0.12	0.0078370	0.38	0.12	0.0238940
PACE1	Protein-associating with the carboxyl-terminal domain of ezrin	0.44	0.09	0.0001115	0.45	0.09	0.0000588
TDPZ4	TD and POZ domain-containing protein 4	0.44	0.11	0.0029837	0.46	0.11	0.0010879
ARHG8	Neuroepithelial cell-transforming gene 1 protein	0.43	0.10	0.0006373	0.36	0.10	0.0053661
KIBRA	Protein kibra	0.43	0.12	0.0087474	0.34	0.12	0.0391711
NUP155	nuclear pore complex Nup155	0.43	0.09	0.0002208	0.28	0.09	0.0246441
CP18A	Cytochrome P450 18a1	0.42	0.10	0.0009586	0.40	0.10	0.0013608
MCTS1	Malignant T-cell-amplified sequence 1 homolog {ECO:0000305}	0.41	0.10	0.0033259	0.35	0.10	0.0128887
E74EB	Ecdysone-induced protein 74EF isoform B	0.41	0.14	0.0444891	0.56	0.14	0.0017739

KMT2D	histone-lysine N-methyltransferase 2D-like isoform X3	0.41	0.10	0.0043781	0.40	0.10	0.0034388
PRPF8	pre-mRNA-processing-splicing factor 8	0.39	0.12	0.0226642	0.36	0.12	0.0289768
KAT3	Kynurenine--oxoglutarate transaminase 3	0.38	0.11	0.0173255	0.38	0.11	0.0129540
RIOK1	Serine/threonine-protein kinase RIO1	0.38	0.10	0.0092736	0.36	0.10	0.0106163
BRN	Beta-1.3-galactosyltransferase brn	0.37	0.11	0.0222078	0.37	0.11	0.0176537
LOC108680771	LOC108680771	0.37	0.11	0.0269895	0.71	0.11	0.0000002
SMC5	Structural maintenance of chromosomes protein 5	0.36	0.12	0.0356370	0.41	0.12	0.0091393
DOT1	histone-lysine N- H3 lysine-79 specific isoform X1	0.36	0.12	0.0385444	0.42	0.12	0.0099559
SPS1	Selenide. water dikinase	0.36	0.09	0.0025903	0.43	0.09	0.0001058
MARS	methionine--tRNA cytoplasmic	0.36	0.09	0.0025918	0.35	0.09	0.0031864
K2013	Uncharacterized protein KIAA2013 homolog	0.36	0.09	0.0046082	0.34	0.09	0.0053300
HSC70	Heat shock cognate 71 kDa protein	0.35	0.09	0.0058374	0.30	0.09	0.0202185
EIF4A1	eukaryotic initiation factor 4A-I-like	0.35	0.09	0.0073410	0.28	0.09	0.0416971
PPAN	Peter pan	0.35	0.09	0.0051407	0.27	0.09	0.0313504
ZN593	Zinc finger protein 593 homolog	0.34	0.11	0.0360686	0.55	0.11	0.0000567
TRM6	tRNA (adenine(58)-N(1))-methyltransferase non-catalytic subunit TRM6	0.32	0.10	0.0312233	0.29	0.10	0.0440302
PMS1	PMS1 protein homolog 1	0.32	0.10	0.0254395	0.29	0.10	0.0352746
ADA10	Disintegrin and metalloproteinase domain-containing protein 10	0.32	0.09	0.0107752	0.33	0.09	0.0048806
NCDN	Neurochondrin homolog	0.30	0.09	0.0235936	0.29	0.09	0.0211709
ALDH2	Aldehyde dehydrogenase. mitochondrial	0.30	0.09	0.0214583	0.33	0.09	0.0053661
PHET	phenylalanine--tRNA ligase beta subunit	0.28	0.09	0.0397577	0.32	0.09	0.0105636
CSN7A	COP9 signalosome complex subunit 7a	0.27	0.09	0.0466937	0.30	0.09	0.0172088
PMM	Phosphomannomutase	0.27	0.09	0.0387830	0.27	0.09	0.0295060
TS101	Tumor susceptibility gene 101 protein	0.27	0.09	0.0454762	0.25	0.09	0.0495440
SNRPF	small nuclear ribonucleo F	-0.28	0.09	0.0410325	-0.35	0.09	0.0035018
GST1	Glutathione S-transferase 1	-0.28	0.09	0.0444891	-0.45	0.09	0.0001058
UHRF1	E3 ubiquitin-protein ligase UHRF1	-0.28	0.09	0.0401129	-0.32	0.09	0.0108091
PSTK	L-seryl-tRNA(Sec) kinase	-0.29	0.09	0.0227280	-0.41	0.09	0.0003323
MRGBP	MRG/MORF4L-binding protein	-0.30	0.09	0.0187662	-0.27	0.09	0.0336118
COPZ1	Coatomer subunit zeta-1	-0.31	0.09	0.0184945	-0.30	0.09	0.0144266
BRC1	Broad-complex core protein isoforms 1/2/3/4/5	-0.31	0.09	0.0226642	-0.35	0.09	0.0048292
RED1	Double-stranded RNA-specific editase 1	-0.32	0.10	0.0239457	-0.28	0.10	0.0429797
GPKOW	G patch domain and KOW motifs-containing protein	-0.33	0.11	0.0352556	-0.61	0.11	0.0000018

NSUN6	Putative methyltransferase NSUN6	-0.33	0.11	0.0444891	-0.35	0.11	0.0224483
UBE2C	Ubiquitin-conjugating enzyme E2 C	-0.34	0.11	0.0329189	-0.40	0.11	0.0046259
MOE	moesin ezrin radixin homolog 1 isoform X2	-0.34	0.09	0.0069359	-0.34	0.09	0.0052855
AGO2	Protein argonaute-2 {ECO:0000255|HAMAP-Rule:MF_03031}	-0.35	0.12	0.0472961	-0.33	0.12	0.0450910
ALDH7A1	alpha-aminoadipic semialdehyde dehydrogenase-like	-0.35	0.09	0.0043781	-0.31	0.09	0.0094807
MRC2	C-type mannose receptor 2	-0.35	0.09	0.0069359	-0.27	0.09	0.0486785
SYWM	Tryptophan--tRNA ligase. mitochondrial	-0.35	0.11	0.0363557	-0.44	0.11	0.0029954
ACUI	Acrylyl-CoA reductase AcuI	-0.35	0.09	0.0043781	-0.40	0.09	0.0005558
VAPA	Vesicle-associated membrane protein-associated protein A	-0.35	0.09	0.0039971	-0.41	0.09	0.0003095
sppA	protease 4-like	-0.35	0.12	0.0472589	-0.48	0.12	0.0019653
PHF20	PHD finger protein 20	-0.36	0.10	0.0122738	-0.31	0.10	0.0284388
RM54	39S ribosomal protein L54. mitochondrial	-0.36	0.10	0.0063316	-0.28	0.10	0.0410041
LDHD	D-lactate dehydrogenase	-0.36	0.11	0.0251847	-0.35	0.11	0.0230781
RAB18	Ras-related protein Rab-18	-0.37	0.10	0.0077840	-0.41	0.10	0.0015848
LGUL	Lactoylglutathione lyase	-0.37	0.09	0.0038386	-0.39	0.09	0.0010422
EIF3I	Eukaryotic translation initiation factor 3 subunit I {ECO:0000255|HAMAP-Rule:MF_03008}	-0.37	0.11	0.0189581	-0.33	0.11	0.0362971
LDB2	LIM domain-binding protein 2	-0.38	0.12	0.0345485	-0.60	0.12	0.0000423
CNOT2	CCR4-NOT transcription complex subunit 2	-0.38	0.09	0.0034656	-0.40	0.09	0.0009202
FCHO2	F-BAR domain only protein 2	-0.38	0.10	0.0034656	-0.49	0.10	0.0000273
AAMP	Angio-associated migratory cell protein	-0.39	0.13	0.0444891	-0.51	0.13	0.0028439
LOC107984796	extensin-1-like	-0.40	0.11	0.0083885	-0.64	0.11	0.0000008
HEXDC	Hexosaminidase D	-0.40	0.10	0.0030982	-0.55	0.10	0.0000031
S12A6	Solute carrier family 12 member 6	-0.40	0.10	0.0040600	-0.44	0.10	0.0007935
COX2	Cytochrome c oxidase subunit 2	-0.40	0.11	0.0076313	-0.63	0.11	0.0000016
ZWILC	Protein zwilch homolog	-0.41	0.10	0.0017125	-0.56	0.10	0.0000017
U5S1	116 kDa U5 small nuclear ribonucleoprotein component	-0.42	0.11	0.0059823	-0.33	0.11	0.0328115
NMD3	60S ribosomal export protein NMD3	-0.42	0.09	0.0004874	-0.49	0.09	0.0000111
PSMG2	Proteasome assembly chaperone 2	-0.43	0.11	0.0068971	-0.39	0.11	0.0123073
C14ORF119	PREDICTED: uncharacterized protein C14orf119 homolog isoform X1	-0.43	0.12	0.0146421	-0.41	0.12	0.0164560
PIPNB	Phosphatidylinositol transfer protein beta isoform	-0.43	0.10	0.0013249	-0.48	0.10	0.0001226
DDX5	Probable ATP-dependent RNA helicase DDX5	-0.43	0.12	0.0116100	-0.40	0.12	0.0172088
B3GALT2	glyco -N-acetylgalactosamine 3-beta-galactosyltransferase 1-like	-0.44	0.11	0.0051407	-0.34	0.11	0.0352532

RFT2	Riboflavin transporter 2	-0.45	0.12	0.0055996	-0.41	0.12	0.0104109
NDKA	Nucleoside diphosphate kinase A	-0.46	0.13	0.0104547	-0.38	0.13	0.0356925
ARF6	ADP-ribosylation factor 6	-0.48	0.13	0.0079195	-0.53	0.13	0.0015662
MAGT1	Magnesium transporter protein 1	-0.48	0.10	0.0002753	-0.40	0.10	0.0030756
LOC100878390	LOC100878390	-0.50	0.09	0.0000064	-0.66	0.09	0.0000000
AGO1	Protein argonaute-1	-0.58	0.15	0.0051407	-0.65	0.15	0.0007739
RIC8A	Synembryn-A	-0.61	0.13	0.0003369	-0.74	0.13	0.0000033
SCYL1	N-terminal kinase-like protein	-0.67	0.15	0.0004038	-0.80	0.15	0.0000057
PRRCA	Protein PRRC1-A	-0.70	0.16	0.0009586	-0.98	0.16	0.0000003
CFDP2	Craniofacial development protein 2	-0.75	0.21	0.0117984	-0.77	0.21	0.0064325
VP13A	Vacuolar protein sorting-associated protein 13A	-0.78	0.25	0.0380698	-0.84	0.25	0.0146542
RAS	Ras-like protein	-0.82	0.18	0.0004272	-0.62	0.18	0.0113938
NEUL4	Neuralized-like protein 4	-0.85	0.28	0.0405955	-0.81	0.28	0.0417874
S35E1	Solute carrier family 35 member E1 homolog	-0.90	0.13	0.0000000	-0.50	0.13	0.0052855
NDUV2	Probable NADH dehydrogenase [ubiquinone] flavoprotein 2. mitochondrial	-0.99	0.22	0.0006739	-0.74	0.22	0.0144266
UBR4	E3 ubiquitin- ligase UBR4	-1.01	0.27	0.0058374	-0.99	0.27	0.0053300
PGBM	Basement membrane-specific heparan sulfate proteoglycan core protein	-1.08	0.18	0.0000009	-0.98	0.18	0.0000072
LOC10953479	LOC10953479	-1.45	0.26	0.0000091	-1.12	0.26	0.0011223
XXYLT1	xyloside xylosyltransferase 1-like	-1.76	0.49	0.0098016	-1.77	0.49	0.0075057
DPH2	2-(3-amino-3-carboxypropyl)histidine synthase subunit 2	-2.16	0.36	0.0000008	-1.94	0.36	0.0000096
IMPA1	Inositol monophosphatase 1	-4.27	0.55	0.0000000	-2.95	0.55	0.0000111

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